Translocation of *Fasciola hepatica* via international livestock movements: Development of ante-mortem molecular diagnostic tools for the identification of *Fasciola* spp. in livestock

A thesis with publication submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Veterinary Science)

by

Nichola Eliza Davies Calvani

BAnVetBioSc (Hons II)

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Supervisor: Associate Professor Russell Bush

Auxiliary supervisor: Professor Jan Šlapeta

Sydney School of Veterinary Science, Faculty of Science

Mekong Livestock Research Group and Laboratory of Veterinary Parasitology

The University of Sydney

Statement of originality

This thesis is submitted to The University of Sydney in order to fulfil the requirements for the degree of Doctor of Philosophy. I, Nichola Eliza Davies Calvani, declare that, to the best of my knowledge, the contents of this thesis is original and that all material herein is my own work, unless acknowledged in the text.

This thesis has not been submitted for any other degree or purposes at The University of Sydney or any other academic institution.

Nichola Calvani

Preface

This thesis comprises six chapters, four of which are peer-reviewed publications where I am the first author (Chapters 2-5). A declaration of my contribution and the contributions of all co-authors has been included at the start of each chapter. Chapter 1 provides an outline of the life cycle of *Fasciola hepatica* and *Fasciola gigantica*, highlights the human and animal health impacts, provides an overview of the available diagnostic methods and introduces the thesis aims, which are subsequently addressed throughout the remainder of the thesis.

Chapter 2 is a published research paper detailing the development and validation of a molecular workflow for the ante-mortem diagnosis of *Fasciola* spp. in faecal samples.

Chapter 3 is a published research paper that compares the time of first detection of *Fasciola hepatica* infection in experimentally-infected sheep using three different methods, including the one developed and described in Chapter 2. The sheep used in this study were part of a herd maintained at Yarrandoo Research and Development Centre (NSW, Australia) by Elanco Animal Health for product development and testing purposes. Animal ethics was approved by the Elanco Australasia Pty Ltd Animal Ethics Committee (approval ELA170004).

Chapter 4 is a published short communication research paper that describes the minimum faecal egg load required for molecular detection of *F. hepatica* in raw cattle and sheep faeces using standard laboratory equipment. This study was conducted with the assistance of several undergraduate students in the Bachelor of Animal and Veterinary Science degree as a part of the requirements of a Laboratory Disease Investigation study and their contributions are duly acknowledged.

Chapter 5 is a published research paper that provides qualitative ante-mortem *Fasciola* spp. differentiation using Taqman probe-based SNP genotyping assays and the first quantitative assessment of the contribution of each *Fasciola* species via Next Generation Sequencing. These methods were used to survey 153 local cattle from an area of Northern Laos with frequent international livestock movements. The collection of these samples and my time spent in Laos was supported by a scholarship provided by Four Season Co. via a Business Partnership Platform (BPP) initiative from The Department of Foreign Affairs and Trade (DFAT).

Chapter 6 provides a discussion of the outcomes of this thesis in the context of the introduction and aims presented in Chapter 1. Contributions to research outcomes, limitations and future directions are also included.

The research presented herein was in-part funded by the Australian Centre for International Agricultural Research (ACIAR). During my candidature I was the recipient of two William and Catherine McIlrath Grants-in-Aid scholarships from The Faculty of Science, The University of Sydney, which provided funding for the fieldwork I conducted in Laos and enabled me to travel to The University of Liverpool, UK to conduct a research exchange and present my work. Funding for fieldwork, national and international conference presentations was provided by The Australian Society for Parasitology (ASP) and the Postgraduate Research Support Scheme (PRSS), The Faculty of Science, The University of Sydney and I am incredibly grateful for the opportunities they have allowed me to pursue.

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Who would have thought that a holiday to Southeast Asia in 2012 would have turned me (to my family's complete and utter bewilderment) into a parasitologist? For that I have to thank Dr Natasha Hamilton, for without her help navigating the complex administration system at The University of Sydney I would never have finished my undergraduate degree in the first place, let alone had the drive and support needed to graduate with honours.

I am, of course, indebted to both the Lao field and lab teams, with whom I have weathered literal storms, and who helped me to collect and process over 1000 faecal samples during my most recent field trip alone. Khop chai deu to Phaivanh, Kong, Borkeo, Phoud, Keysone, Boun My, Vilay and all the Lao farmers and their families who were always so cheerful and accommodating. A particular thank you to Dr Syseng and Dr Sothoueun, for their support and facilitation of all our in-country work. Speaking of Laos, an enormous thank you to Franny Earp, student turned field work aficionado, for the emotional and physical support during what would have otherwise been a lonely five months – a long overdue mojidoo when you return. Finally, my thanks to Emeritus Professor Peter Windsor, Katherine Ashley, Isabel MacPhillamy, Luisa Olmo and everyone else in the Mekong Livestock group for your advice and wisdom.

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the Department of Infection Biology at The University of Liverpool for hosting me and teaching me the proper way to eat scones. Although daunting, it was wonderful to be surrounded by so many fluke experts.

To my supervisors, Associate Professor Russell Bush and Professor Jan Šlapeta, thank you for your continued support and mentorship throughout my honours and PhD years. While it was sometimes tricky to manage your competing interests and opinions, collectively you have shown me the full picture of research and its impacts, providing me with an enviable combination of skills and experiences as a result. Through your guidance and encouragement you have helped me to find my confidence both in the lab and in the field. You have not only taught me the value of rigorous scientific enquiry, but also to persevere and adapt when things don't go as planned. Above all else, you have allowed me to make this work my own, all while putting up with my many (many) awful parasite puns. I must thank Jan in particular for his unwavering support and for helping to guide me through a diverse variety of (mis)adventures. Your infectious enthusiasm has provided me with opportunities far beyond the scope of this thesis and is the thing I will miss the most about my time at The University of Sydney.

To my family, while you might not be entirely sure what I do or why I do it, your support throughout has been invaluable. Thank you for sitting through endless stories about my antics in the field and the lab during dinner, for editing countless draft manuscripts and presentations, and for being understanding during my extended absences. I'm sorry to say that while some of those things may change going forward, the poo stories during mealtimes are likely to continue. To Charlie, Annie and Oscar, thank you for keeping my lap warm and my morale up during the many hours spent alone at my computer – you will always be my favourite co-authors. And finally, to Harry, for not only keeping my glass full and our house clean while I was busy working, but for the countless hours spent listening to my stories, complaints, theories and excerpts of my writing in its various forms, as well as for the constant support from near and far – thank you.

Publications, presentations and awards arising from candidature

Publications

Publications featured in this thesis

- Calvani, N. E. D., Ichikawa-Seki, M., Bush, R. D., Khounsy, S., Šlapeta, J. (2020). Which species is in the faeces at a time of global livestock movements: SNP genotyping assays for the differentiation of *Fasciola* spp. *International Journal for Parasitology*, 50 (2), 91-101. doi: http://dx/doi.org/10.1016/j.ijpara.2019.12.002

- Calvani, N. E. D., Cheng, T., Green, C., Hughes, P., Kwan, E., Maher, E., Bush, R. D., Šlapeta, J. (2018). A quick and simple benchtop vortex egg-disruption approach for the molecular diagnosis of *Fasciola hepatica* from ruminant faecal samples. *Parasitology Research*, 117 (8), 2685-2688. doi: http://dx.doi.org/10.1007/s00436-018-5926-3

- Calvani, N. E. D., George, S. D., Windsor, P. A., Bush, R. D., Šlapeta, J. (2018). Comparison of early detection of *Fasciola hepatica* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation. *Veterinary Parasitology*, 251, 85-89. doi: http://dx.doi.org/10.1016/j.vetpar.2018.01.004

- Calvani, N. E. D., Windsor, P. A., Bush, R. D., Šlapeta, J. (2017). Scrambled eggs: A highly sensitive molecular diagnostic workflow for *Fasciola* species specific detection from faecal samples. *PLoS Neglected Tropical Diseases*, 11, e0005931. doi: http://dx.doi.org/10.1371/journal.pntd.0005931

Publications arising from collaborative work

- Francis, E.K., McKay-Demeler, J., Calvani, N.E.D., McDonell, D., Šlapeta, J. (2020). Which larvae are they? Use of single larva for the molecular confirmation of *Cooperia pectinata* and *Cooperia punctata* in Australian cattle. *Veterinary Parasitology*, in press.

- Rush, G., Reynolds, M. W., Calvani, N. E. D., Šlapeta, J. (2019). Addressing the constraints of *Tritrichomonas foetus* sample collection in remote areas: lyophilized modified Diamond's media as a substitute for liquid medium. *Parasitology*, 12, 1-4. doi: http://dx.doi.org/10.1017/S0031182019000258

- Panetta, J. L., Sima, R., Calvani, N. E. D., Hajdusek, O., Chandra, S., Panuccio, J., Šlapeta, J. (2017). Reptile-associated *Borrelia* species in the goanna tick (*Bothriocroton undatum*) from Sydney, Australia. *Parasites and Vectors*, 10, 616-629. doi: http://dx.doi.org/10.1186/s13071-017-2579-5

Presentations

International conference presentations

- Calvani, N. E. D., Van Galen, G., Hughes, K. J., Beasley, A., Shamsi, S. and Šlapeta, J. (July, 2019). *Teaching horse parasitology in the 21st century: An adaptive learning platform*. Work presented at the 27th International Conference for the World Association for the Advancement of Veterinary Parasitology (WAAVP). Madison, USA. (15-minute oral presentation).

- Calvani, N. E. D., Bush, R. D., Šlapeta, J. (July, 2019). *Who's who in the poo: A Taqman assay for the differentiation of* Fasciola hepatica, Fasciola gigantica *and co-infection in ruminant faecal samples.* Work presented at the 27th International Conference for the World Association for the Advancement of Veterinary Parasitology (WAAVP). Madison, USA. (15-minute oral presentation).

- Calvani, N. E. D., Bush, R. D., Šlapeta, J. (September, 2018). *A flexible molecular diagnostic method for the diagnosis of* Fasciola *spp. in ruminant faecal samples*. Work presented at the Molecular and Cellular Biology of Helminth Parasites XI conference. Hydra, Greece. (Poster and 2-minute oral presentation).

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Local conference presentations

- Calvani, N. E. D., Ichikawa-seki, M., Bush, R. D., Šlapeta, J. (July, 2019). *Who's who in the poo: A Taqman assay for the differentiation of* Fasciola hepatica, Fasciola gigantica *and co-infection in ruminant faecal samples.* Work presented at the Sydney School of Veterinary Science Annual Postgraduate Student Conference. Camden, NSW. (8-minute oral presentation).

- Calvani, N. E. D., Van Galen, G., Hughes, K. J., Beasley, A., Shamsi, S. and Šlapeta, J. (July, 2019). *Educating digital natives on equine parasitology: a multi-institutional collaborative approach*. Presented by A. Beasley on behalf of N. E. D. Calvani at the Australian Society for Parasitology's (ASP) 2019 international conference. Adelaide, SA. (10-minute oral presentation).

- Calvani, N. E. D., Bush, R. D., Šlapeta, J. (November, 2018). *A flexible molecular diagnostic method for the diagnosis of* Fasciola *spp. in ruminant faecal samples*. Work presented at the Sydney School of Veterinary Science Annual Postgraduate Student Conference. Sydney, NSW. (Poster and 1-minute oral presentation).

- Calvani, N. E. D., Bush, R. D., Šlapeta, J. (September, 2018). *A flexible molecular diagnostic method for the diagnosis of* Fasciola *spp. in ruminant faecal samples*. Work presented at the Australian Society for Parasitology's (ASP) 2018 international conference. St Kilda, VIC. (Poster and 1-minute oral presentation).

- Calvani, N. E. D., Šlapeta, J. (September, 2018). *"Learning veterinary parasitology without even feeling like you are learning"*. Work presented at the Australian Society for Parasitology's (ASP) 2018 international conference. St Kilda, VIC. (10-minute oral presentation).

- Calvani, N. E. D., Windsor, P. A., Bush, R. D., Šlapeta, J. (November, 2017). *Scrambled eggs: A highly sensitive molecular diagnostic workflow for* Fasciola *species specific detection from faecal samples*. Paper presented at the Sydney School of Veterinary Science Annual Postgraduate Student Conference. Sydney, NSW. (Poster and 1-minute oral presentation).

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highly sensitive molecular diagnostic workflow for Fasciola *species specific detection from faecal samples*. Paper presented at the Australian Society for Parasitology (ASP) Annual Conference. Leura, NSW. (Poster and 1-minute oral presentation).

- Calvani, N. E. D., Sothoeun, S., Šlapeta, J., Bush, R. D., Windsor, P. A. (November, 2016). *The importance and prevalence of* Fasciola gigantica *in smallholder cattle herds in Cambodia*. Work presented at the Marie Bashir Institute (MBI) Colloquium. Sydney, NSW. (Poster).

- Calvani, N. E. D., Bush, R. D., Šlapeta, J. (November, 2016). *Investigating* Fasciola *spp. in smallholder cattle herds in Cambodia*. Work presented at the Faculty of Veterinary Science Postgraduate Student Conference. Sydney, NSW. (10-minute oral presentation).

Invited presentations

- Calvani, N.E.D., Bush, R.D., Šlapeta, J. (November, 2018). *Diagnostic workflows for fluke surveillance*. Invited speaker at the Elizabeth Macarthur Agricultural Institute (EMAI) and University of Sydney Seminar Day. Menangle, NSW, Australia.

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Contribution to the scientific community

- Supervisor of Animal and Veterinary Bioscience undergraduate students in Laboratory Disease Investigation at the Sydney School of Veterinary Science, The University of Sydney (2019). Project: Streamlining the molecular diagnosis of avian pathogens.

- Invited manuscript reviewer, Biologia (2019), Veterinary Parasitology (2019), International Journal for Parasitology (2018-2019) and Trends in Parasitology (2018).

- Associate supervisor for research honours student Simin Khan at the Sydney School of Veterinary Science, The University of Sydney (2018). Project: Smallholder farmer knowledge, attitudes and practices regarding the intermediate host of *Fasciola gigantica*, *Lymnaea* snail species in Lao PDR.

- Supervisor of Animal and Veterinary Bioscience undergraduate students in Laboratory Disease Investigation at the Sydney School of Veterinary Science, The University of Sydney (2018). Project: *Capillaria aerophila* in Australian foxes.

- Associate supervisor of Animal and Veterinary Bioscience undergraduate students in Laboratory Disease Investigation at the Sydney School of Veterinary Science, The University of Sydney (2017). An alternative egg homogenisation method for the extraction of *Fasciola* spp. DNA from ruminant faecal samples.

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- William and Catherine McIlrath Grants-in-Aid scholarship. Faculty of Science, The University of Sydney (July, 2019). Received to fund data collection and field work in Northern Laos.

- Recipient of a Department of Foreign Affairs and Trade (DFAT) Business Partnerships Platform (BPP) PhD Scholarship (July 2017 – November 2019).

- Postgraduate Research Support Scheme (PRSS). Faculty of Science, The University of Sydney (2016 – 2019).

- Awarded best presentation (mid-candidature category) at the 2018 Sydney School of Veterinary Science Annual Postgraduate Student Conference, The University of Sydney (November, 2018).

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- William and Catherine McIlrath Grants-in-Aid scholarship. Faculty of Science, The University of Sydney (July, 2018). Received to support a research exchange at the Department of Infection Biology, The University of Liverpool, Liverpool UK.

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- Invited as a featured author in PLoS redditscience Wednesday “Ask Me Anything” session (2017)

Thesis abstract

Fasciolosis caused by infection with *Fasciola hepatica* and *Fasciola gigantica* is a re-emerging zoonotic disease of worldwide importance. Of the two species, *F. hepatica* is considered the greater human and animal health risk, with an estimated 91 million people considered at risk of infection. Livestock losses are estimated to exceed US $3 billion/year and are of particular concern in developing countries where aquatic rice production coupled with large ruminant husbandry provides the ideal habitat for parasite proliferation. While *F. hepatica* and *F. gigantica* have temperate and tropical distributions, respectively, parasite sympatry occurs in parts of Asia, Africa and the Middle East. Recent increases in the demand for animal-derived protein in countries such as China and Vietnam, has seen consistent growth in the international trade of livestock from *F. hepatica*-endemic countries. Translocation of *F. hepatica* into the region is particularly concerning in the Lao People's Democratic Republic, which acts as a major livestock thoroughfare in the region. Despite these human and animal health impacts, no test is capable of ante-mortem *Fasciola* spp. differentiation. The aim of this thesis was to design, validate and deploy a suite of highly sensitive molecular diagnostic tools for *Fasciola* spp. differentiation from faecal samples to enable ante-mortem screening of livestock in Northern Laos. In combination with a traditional sedimentation method and with the use of a high-speed benchtop homogeniser, the detection and quantification of *Fasciola* spp. infection in 100% of cattle with low faecal egg loads (<25 EPG) was possible. This workflow was then used to investigate the point of first detection of *F. hepatica* infection in experimentally-infected sheep and compare the results to a traditional sedimentation and commercially-available coproantigen ELISA. Faecal samples were first considered positive at 6, 7 and 8 weeks post infection (WPI) by coproELISA, real-time PCR and sedimentation, respectively, and by 9 WPI 100% of samples were positive by all three methods. To increase sample throughput a simplified method using un-concentrated faecal samples was developed and a standard benchtop vortex was used in lieu of the high-speed homogeniser. The limit of detection using this method was 10 and 20 EPG for sheep and cattle, respectively, when mechanical disruption was extended from 40 seconds to 5 minutes using the benchtop vortex. Finally, several single nucleotide polymorphism assays targeting key ITS-1 and LSU rDNA residues were developed for the differentiation of *Fasciola* spp. in faecal samples via conventional and real-time PCR, as well as a quantitative Next Generation Sequencing method to determine the contribution of nucleotides from each species. These assays were applied to 153 faecal samples collected from local cattle across 27 villages in Northern Laos to detect *F. hepatica* translocation in an area of SE Asia with frequent international livestock trade. Of the 91 positive samples, 11 were identified as containing *F. hepatica* DNA, indicating establishment of the *F. hepatica* life cycle in Northern Laos.

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List of abbreviations used

µL	Microlitre
µm	Micrometre
ACIAR	Australian Centre for International Agricultural Research
AD	Allelic discrimination
ASV	Amplicon sequence variant
BCS	Body condition score
bp	Base pair
BPP	Business Partnership Platform
coproELISA	Coprological enzyme-linked immunosorbent assay
Ct	Cycle threshold
DFAT	Department of Foreign Affairs and Trade
DNA	Deoxyribonucleic acid
DOI	Digital Object Identifier
DPI	Days post infection
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram (of faeces)
EtOH	Ethanol
FEC	Faecal egg count
g	Gram
ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
Lao PDR	Lao People's Democratic Republic
lsRNA	Large subunit ribosomal ribonucleic acid
LSU	Large subunit
m/s	Metres per second
mEPG	Morphological eggs per gram of faeces based on traditional faecal egg counts
Min	Minute
mtDNA	Mitochondrial DNA

NEJ	Newly excysted juvenile
ng	Nanogram
NGS	Next Generation Sequencing
NSW	New South Wales
nt	Nucleotide
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PPP	Pre-patent period
PRSS	Postgraduate Research Support Scheme
qEPG	Molecular estimate of eggs per gram of faeces based on qPCR standard curves
qPCR	Quantitative polymerase chain reaction
RFU	Relative fluorescence unit
SD	Standard deviation
Se	Sensitivity
SNP	Single nucleotide polymorphism
Sp	Specificity
SSVS	Sydney School of Veterinary Science
USDA	United States Department of Agriculture
VIC	Victora
WHO	World Health Organisation
WPI	Weeks post infection

Chapter 1

Introduction:
Background, knowledge gaps and thesis aims

1.1 Fasciolosis – a neglected disease of global importance

Fasciolosis is a zoonotic disease caused by infection with the digenean trematodes *Fasciola hepatica* and *Fasciola gigantica*. The global distribution of these parasites is associated with post-domestication livestock movements and trade (Mas-Coma et al., 2009). *F. hepatica* is primarily located in temperate regions, while *F. gigantica* is restricted to tropical areas and parasite sympatry occurs in parts of Africa and Asia where these climatic zones overlap (Mas-Coma et al., 2009). Infection with *F. hepatica* is well-recognised as a significant constraint to livestock production in the developed world (Charlier et al., 2014; Howell et al., 2015; Beesley et al., 2018). The human and animal health impacts due to infection with *F. gigantica*, however, are often overlooked resulting in its classification as a "Neglected Tropical Disease" by the World Health Organisation (WHO, 2019).

Over 91 million people are considered at risk of infection, most being women and children (Keiser and Utzinger, 2005). Human-endemic areas are found in China, Vietnam, Egypt, Portugal, Spain, Iran and parts of South America, with many cases likely to be under-reported due to the limited medical facilities available throughout the developing world (Mas-Coma et al., 1999; Mas-Coma et al., 2009). Areas where human infections have been recorded are classified as being either autochthonous isolated, imported or hypo-, meso- and hyper-endemic (Mas-Coma, 2005). These classifications are based on a combination of factors including parasite prevalence, level of egg shedding by infected hosts and hygiene and sanitation practices contributing to the exposure of definitive hosts to infective stages (Mas-Coma, 2005; Mas-Coma et al., 2009).

1.2 The *Fasciola* spp. life cycle

The life cycle is indirect, requiring both a mammalian definitive host and a freshwater aquatic snail intermediate host for completion (Figure 1) (Thomas, 1883). The definitive hosts, usually sheep or cattle, become infected by ingestion of metacercariae that are either encysted on vegetation and pasture, or free-floating in water (Thomas, 1883; Wright, 1927). Excystation occurs in the small intestine within an hour of ingestion where the newly excysted juveniles (NEJs) burrow through the intestinal wall into the abdominal cavity (Dixon, 2009). From here, the NEJs move towards the liver, eventually penetrating the liver capsule 4-6 days post infection (Dawes and Hughes, 1964; Boray, 1969). The immature flukes migrate through the liver for 5-6 weeks in the case of *F. hepatica* in sheep, or for up to 11 weeks for *F. gigantica* in cattle (Dawes and Hughes, 1964; Guralp et al., 1964; Boray, 1967). This process of migration and maturation results in extensive damage, haemorrhage and fibrosis of the liver, and is the cause of most clinical signs (Boray, 1969). At the completion of the pre-patent period (PPP) the mature flukes reach the bile ducts, either 8-12 weeks post-infection (WPI) for *F. hepatica*, or 12-16 WPI for *F. gigantica*, where they commence egg laying (Boray, 1969; Prasitirat et al., 1996; Dalton, 1999).

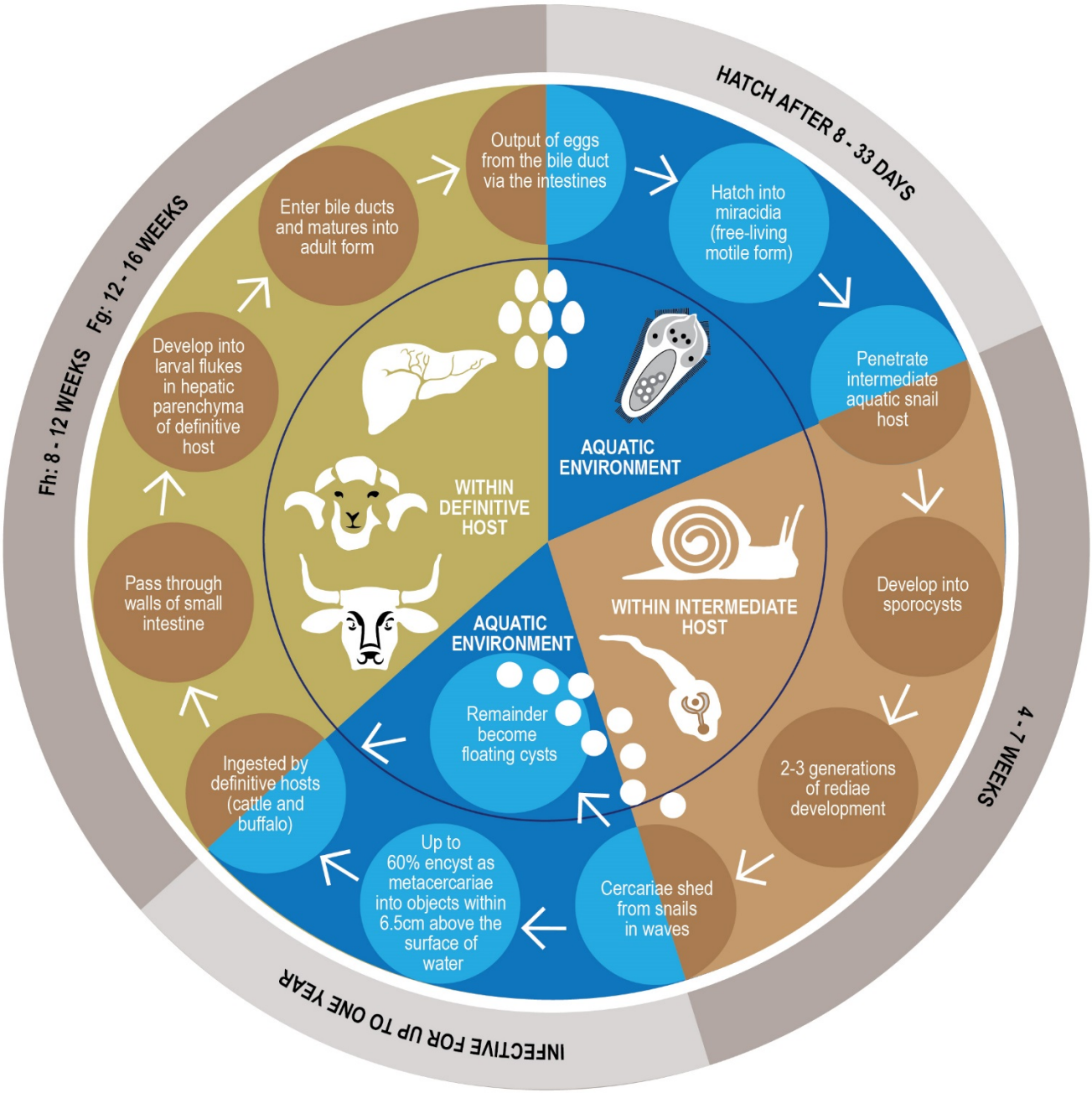

Figure 1. A summary of the life cycle of *Fasciola hepatica* (Fh) and *Fasciola gigantica* (Fg). Figure based on the life cycle as elucidated by Thomas (1883) and summarised in *Fasciolosis* (Dalton, 1999).

Adult flukes are hermaphroditic and are capable of both parthenogenetic and sexual reproduction (Dalton, 1999; Hanna et al., 2008). Eggs are shed via the bile ducts into the gastrointestinal tract where they exit the mammalian host into the environment (Thomas, 1883). Embryonation and the subsequent hatching of eggs occurs when they are free from faeces and exposed to light, and must be in the presence of water for infection of the intermediate snail host (Rowcliffe and Ollerenshaw, 1960). Prior to hatching, the development of *F. hepatica* miracidium in the eggs takes 2-3 weeks between 23-26°C (Thomas, 1883). Development is temperature-dependent and can occur in as little as 8 days at 30°C, but may take up to 6 months at 10°C (Rowcliffe and Ollerenshaw, 1960). This process is slightly longer and requires higher temperatures in the case of *F. gigantica*, where hatching occurs in 10-11 days at 37-38°C, or up to 33 days at 17-22°C (Grigoryan, 1958; Guralp et al., 1964). Egg mortality occurs above 37°C for *F. hepatica* and above 43°C for *F. gigantica* (Grigoryan, 1958; Rowcliffe and Ollerenshaw, 1960). The eggs of *F. hepatica* are resistant to freezing and can withstand temperatures as low as -15°C for 24 hours, but are highly sensitive to desiccation and thus require water for embryonation (Ollerenshaw, 1971).

Newly-hatched miracidia are highly mobile but short-lived, and must seek out and infect an intermediate snail host within 24 hours (Thomas, 1883). Various aquatic *Lymnaea* snail species act as intermediate hosts, most of which prefer shallow fresh-water pools and ponds, and are capable of aestivation and hibernation in mud when these areas dry up or when the temperature becomes too cool (Ollerenshaw, 1959; Vázquez et al., 2018). *Lymnaea* snail species implicated in the *Fasciola* spp. life cycle have been shown to have a high capacity for translocation and adaptation to new environments via active and passive migration, with the establishment of new populations aided by their high fecundity (Boray, 1964).

Miracidia infect snails via penetration of the foot, mandible or tentacles, after which they migrate to the digestive gland (liver) of the snail as sporocysts (Dawes, 1959). Here the sporocysts undergo clonal expansion producing rediae from which cercariae are later produced via another round of clonal expansion (Thomas, 1883). This process is complex and ongoing, with rediae able to produce both cercariae and daughter rediae. Cercariae emerge from infected snails 4-7 WPI (Kendall and McCullough, 1951). Their tadpole-like tails provide motility in water where they encyst on plants and nearby vegetation any time between two minutes to two hours post-emergence from their intermediate snail hosts (Thomas, 1883; Wright, 1927). After encystation they become metacercariae and are almost immediately infective to mammalian definitive hosts (Boray, 1969).

Infective metacercariae are the most resistant stage of the life cycle and may survive in the environment for over a year with adequate moisture (Boray, 1969). Metacercariae of *F. hepatica* have been shown to remain infective after storage at -2°C for up to eight weeks, while metacercariae of *F. gigantica* are far more sensitive to low temperatures, with their viability rapidly decreasing below 21°C (Boray,

1969). The infectivity of metacercariae of both species is affected by higher temperatures, although the upper limits of survival are extended when adequate moisture is maintained (Boray, 1969).

All stages of *Fasciola* spp. are highly prolific, as are their intermediate snail hosts. Adult *F. hepatica* may lay up to 24,000 eggs per day, and have been known to persist in the gall bladder and bile ducts of infected sheep for over 11 years (Durbin, 1952; Happich and Boray, 1969). Well-fed snails infected with up to ten rediae are capable of producing over 2000 cercariae each, enabling rapid expansion of populations when infected snails are translocated to new environments (Kendall and Ollerenshaw, 1963).

1.3 Clinical signs and production impacts

Livestock disease as a result of infection is either acute, sub-acute or chronic depending on the number of parasites ingested (Boray, 196; Boray, 1969). Production impacts are associated with the damage caused by immature stages as they migrate through the liver. Weight loss, decreased body condition scores (BCS) and reduced milk yield are typical of sub-acute and chronic infections (Hope Cawdery et al., 1977; Howell et al., 2015; Köstenberger et al., 2017; Mazeri et al., 2017; da Costa et al., 2019). Other related production impacts, such as increased time to sexual maturity and longer inter-calving intervals, are also seen (Hope Cawdery, 1976; Charlier et al., 2007). In developing countries infected cattle and buffalo show decreased draught power and fewer days in work, likely due to anaemia caused by damage to the liver from the migration of immature flukes (ACIAR, 2008). In acute infections, where very large numbers of the infective stages are ingested over a short period, sudden death in the absence of clinical signs may occur (Boray, 1969). Sheep are the primary definitive hosts of *F. hepatica*, while large ruminants, such as cattle and buffalo, are more commonly infected with *F. gigantica* (Dawes and Hughes, 1964; Boray, 1969; Mas-Coma et al., 2009). When comparing individual adult flukes, *F. gigantica* may be considered more pathogenic than *F. hepatica* due to their larger size and longer PPP, resulting in more time spent migrating through and damaging the liver of infected hosts (Valero et al., 2016). However, infection with *F. hepatica* typically occurs in much higher numbers than *F. gigantica*, with more flukes inflicting damage on the liver of definitive hosts (Boray, 1967; Dalton, 1999). Infection with *F. hepatica* has also been shown to modulate the host's immune response, leaving them more prone to infection with other pathogens, such as *Bordetella pertussis* and *Mycobacterium bovis* (Brady et al., 1999; Naranjo Lucena et al., 2017).

In human infections, as with infection in animals, clinical signs depend on the period of infection (invasive/acute or chronic) and are related to the level of damage to the liver (Mas-Coma, 2005). Ectopic or aberrant fasciolosis occurs when migrating immature flukes find their way to other organs, most commonly elsewhere in the gastrointestinal tract, such as the pancreas or spleen, but also the abdominal wall, heart, lungs and, occasionally, the eyes or brain (Arjona et al., 1995; Mas-Coma et al., 2014). Reports of infection with *F. hepatica* are more common in human cases than infection with *F. gigantica*, leading to

the assumption that *F. hepatica* has the greater zoonotic potential. The case may be, however, that human cases of fasciolosis caused by infection with *F. gigantica* are under-reported due to their occurrence in tropical developing countries where limited access to medical care prevents diagnosis and disease reporting.

1.4 *Fasciola* spp. hybrids

Hybridisation between *F. hepatica* and *F. gigantica* is being increasingly reported in the literature, with hybrid hotspots arising throughout Asia (Agatsuma et al., 2000; Le et al., 2008; Le et al., 2009; Peng et al., 2009; Nguyen et al., 2012). However, very little is known about the biology and functional implications of *Fasciola*-hyrbids and their potential for introgression. There are fears, however, that hybrid vigour may result in intermediate phenotypes that are larger than traditional *F. hepatica* adults while retaining their high pathogenicity and growing capacity for anthelmintic resistance (Overend and Bowen, 1995; Itagaki et al., 2011; Brockwell et al., 2014).

The experimental infection of Saanen goats with both *F. hepatica* and *F. gigantica* has provided evidence of hybridisation in co-infected definitive hosts (Itagaki et al., 2011). In order to examine the viability of successive hybrid generations and explore potential reproductive isolation, metacercariae from the parental generation were fed to two populations of Wistar rats resulting in the production of F1 and F2 *Fasciola*-hybrids. The results demonstrate that, although there is decreased egg development and therefore a lower hatching rate in later generations, adults of the F1 generation maintain the capacity for normal spermatogenesis, and show that both the F1 and F2 generations reach full maturity in their respective definitive hosts (Itagaki et al., 2011). Adult *F. hepatica* and *F. gigantica* are known to self-fertilise and reproduce via parthenogenesis, the impacts of which on the generation and maintenance of hybrid populations is unknown. Regardless of their ability to survive beyond the F2 generation, continued reports of their occurrence throughout the literature and the many unanswered questions about their biology, infectivity and pathogenicity warrants further investigation.

1.5 Detection of infection and *Fasciola* spp. differentiation in livestock

Several methods of *Fasciola* spp. detection have been developed for use in livestock (Table 1) (Happich and Boray, 1969; Valero et al., 2009b). However, most are limited by their extended PPP, making early diagnosis difficult, and none are capable of rapid and robust *Fasciola* spp. differentiation. Traditionally, infections are diagnosed via sedimentation and counting of eggs from faeces (Happich and Boray, 1969). While this method is low-cost, it is time-consuming and has limited sensitivity, particularly in large ruminants where the increased faecal volume dilutes the number of eggs on offer for detection. It is also difficult to differentiate *Fasciola* spp. infections in areas of parasite sympatry using traditional sedimentation methods, where factors such as host species, age and health status results in overlapping egg morphology (Valero et al., 2009a).

Immunologic techniques, including commercialised antibody and coprological antigen ELISAs, have been developed for earlier detection of *Fasciola* spp. infection before completion of the PPP (Salimi-Bejestani et al., 2007; Valero et al., 2009b). Although more expensive than a traditional sedimentation, the antibody ELISA enables screening of large numbers of animals via the use of bulk milk tank samples (Salimi-Bejestani et al., 2005). This method is capable of detecting infection in naïve sheep as early as four WPI (Valero et al., 2009b). The maintenance of positive antibody titres post-treatment, however, limits the application of the antibody ELISA to the detection of *Fasciola* spp. exposure (Sánchez-Andrade et al., 2001; Brockwell et al., 2013).

The coprological antigen ELISA (coproELISA) enables the diagnosis of current infections via the detection of *F. hepatica* and *F. gigantica* antigens as early as six and nine WPI, respectively, with antigen levels declining rapidly post-treatment (Mezo et al., 2004). However, as with the traditional sedimentation methods, it is limited in its ability to diagnose infection in large ruminants due to antigen dilution in their increased faecal volumes (Martinez-Sernandez et al., 2016). Regardless of their capacity to diagnose large numbers of animals earlier in infection than traditional methods, neither the antibody ELISA nor the coproELISA enable *Fasciola* species identification.

Molecular tools are increasingly being used for *Fasciola* spp. differentiation, but rely on access to adult specimens collected post-mortem or the detection of infection in snails (Marcilla et al., 2002; Itagaki et al., 2005; Ai et al., 2010; Ai et al., 2011; Arifin et al., 2016;). A lack of molecular methods for the diagnosis of fasciolosis in definitive hosts is largely due to a perceived difficulty in rupturing the robust eggs, preventing access to the genetic material within (Ai et al., 2010). This apparent limitation has contributed to the omission of *Fasciola* spp. in molecular panels designed for the diagnosis of a variety of other economically-important gastrointestinal parasites of livestock (Roeber et al., 2017a; Roeber et al., 2017b).

The development of molecular methods allowing highly-sensitive ante-mortem differentiation of *Fasciola* species may provide an alternative diagnostic solution to existing methods, particularly in areas of parasite sympatry. Aside from the ability to provide species confirmation, molecular tools are highly sensitive, which may help to address issues associated with the diagnosis of fasciolosis in large ruminants. This is especially important in cattle and buffalo with chronic infections, where faecal egg output is usually low (<20 EPG) (Boray, 1969; Happich and Boray, 1969). Ante-mortem molecular tools would also allow for the extension of epidemiological investigations beyond the abattoir, thus helping to elucidate information at the source of infection.

Method	Point of first detection	Pros	Cons
Sedimentation & faecal egg count[1]	• *F. hepatica*: 8 – 12 WPI • *F. gigantica*: 12 – 16 WPI	• Low cost • Only basic laboratory equipment required	• Labour-intensive and time-consuming • Overlapping egg morphology prevents species differentiation in areas of parasite sympatry[4] • Decreased sensitivity in light infections (particularly cattle)[5]
Coprological antigen ELISA[2]	• *F. hepatica*: 6 WPI • *F. gigantica*: 9 WPI	• Provides detection of current infection prior to the completion of the PPP (earlier than a traditional sedimentation)	• Decreased sensitivity in light infections (particularly cattle)[5] • Unable to differentiate between species • Laboratory with ELISA capacity required
Antibody ELISA[3]	• Naïve animals: 4 WPI	• The earliest method for detection of infection in naïve animals • The use of bulk milk samples allows the screening of larger sample sizes[6]	• The maintenance of positive antibody titres post-treatment limits its application to the detection of exposure only, cannot differentiate current from past infections[5] • Unable to differentiate between species • Laboratory with ELISA capacity

Table 1. A comparison of different methods for the diagnosis of *Fasciola* spp. in ruminants.

[1]Happich and Boray, (1969), [2]Mezo et al. (2004), [3]Valero et al. (2009b), [4]Valero et al. (2009a), [5]Brockwell et al. (2013), [6]Salimi-Bejestani et al. (2005).

1.6 Knowledge gaps and thesis aims

Diagnostic tools enabling ante-mortem *Fasciola* spp. differentiation are of particular value in areas such as Southeast Asia, where increased demand for animal-derived protein has seen an associated growth in the importation of livestock from *F. hepatica*-endemic countries (USDA, 2019). Establishment of the *F. hepatica* life cycle via the importation of infected animals into an *F. gigantica*-endemic area may give rise to increased incidences of co-infection, and thus, more opportunity for hybridisation between these two species (Shalaby et al., 2013). Despite the significant human and animal health implications, the role of the international livestock trade in the potential translocation of these parasites from endemic to non-endemic areas and the impacts of *Fasciola*-hybrids on definitive hosts remains unexplored, in part due to a lack of diagnostic tools capable of species differentiation.

The overarching aim of this thesis is to investigate the potential translocation of *F. hepatica* into Southeast Asia via the development of molecular tools capable of ante-mortem *Fasciola* spp. diagnosis and species differentiation. The specific objectives to help achieve this aim are:

Aim 1. To develop a molecular diagnostic workflow for the highly-sensitive detection of *Fasciola* spp. DNA from within liver fluke eggs in ruminant faecal samples. The result is the first method available for the robust rupture of *Fasciola* spp. eggs, which was compared to existing methods for *Fasciola* spp. diagnosis, including a commercially-available coproELISA and traditional sedimentation and faecal egg count (Chapter 2, *PLoS Neglected Tropical Diseases* 2017). The point of first detection of *F. hepatica* infection in experimentally-infected Merino sheep using this molecular diagnostic workflow was then examined and compared to the coproELISA and traditional sedimentation (Chapter 3, *Veterinary Parasitology* 2018). The molecular diagnostic workflow was later adapted for increased speed and accessibility via the substitution of a high-speed benchtop homogeniser for the more commonly available standard vortex (Chapter 4, *Parasitology Research* 2018, Short Communication).

Aim 2. To design and validate nucleic acid-based molecular assays capable of *Fasciola* spp. differentiation directly from ruminant faecal samples. The outcome is a suite of conventional and real-time PCR assays utilising multi-copy ribosomal DNA markers, that when combined with the above workflow, are capable of screening ruminant faecal samples for *Fasciola* spp. differentiation beyond the abattoir. The limitations of traditional Sanger sequencing for the identification of *Fasciola*-hybrid adults are highlighted, and suggest the potential misidentification and subsequent under-representation of *Fasciola*-hybrids in the literature. The final result is the development of primers for Next Generation Sequencing (NGS), allowing a quantitative demonstration of the contributions of nucleotides from each *Fasciola* species in what is the first application of this technology to this species (Chapter 5, *International Journal for Parasitology* 2020).

Aim 3. To deploy the above-described molecular assays for ante-mortem diagnosis and differentiation of *Fasciola* spp. in local cattle in Northern Laos, an area of Southeast Asia with frequent international livestock movements. The final outcome of this thesis is the demonstration of *F. hepatica* DNA in Northern Laos as a result of screening 153 local cattle from 27 villages. This finding suggests the establishment of the life cycle in this region, which is likely to have been assisted by the importation of cattle from *F. hepatica*-endemic countries (Chapter 5, *International Journal for Parasitology* 2020).

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Chapter 2

Scrambled eggs: a highly sensitive molecular diagnostic workflow for *Fasciola* species specific detection from faecal samples

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Nichola Calvani in the preparation and submission of the following manuscript:

> Calvani, N.E.D., Windsor, P.A., Bush, R.D. and Šlapeta, J. (2017). Scrambled eggs: A highly sensitive molecular diagnostic workflow for *Fascicola* species specific detection from faecal samples. PLoS Neglected Tropical Diseases 11 (9) e0005931.

Contribution to the manuscript of all authors is as follows:

Task	**Contributing Co-authors**
Conceptualisation	NEDC, JŠ
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Writing of the original draft	NEDC
Critical review and editing of the manuscript	NEDC, PAW, RDB, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct. I have sighted email or other correspondences from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Kind regards,

Assoc. Prof. Russell Bush
14th January 2020

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Scrambled eggs: A highly sensitive molecular diagnostic workflow for *Fasciola* species specific detection from faecal samples

Nichola Eliza Davies Calvani[1,2], Peter Andrew Windsor[2], Russell David Bush[2], Jan Šlapeta[1]*

1 Laboratory of Veterinary Parasitology, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, New South Wales, Australia, **2** Mekong Livestock Research Group, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, New South Wales, Australia

* jan.slapeta@sydney.edu.au

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Abstract

Background

Fasciolosis, due to *Fasciola hepatica* and *Fasciola gigantica*, is a re-emerging zoonotic parasitic disease of worldwide importance. Human and animal infections are commonly diagnosed by the traditional sedimentation and faecal egg-counting technique. However, this technique is time-consuming and prone to sensitivity errors when a large number of samples must be processed or if the operator lacks sufficient experience. Additionally, diagnosis can only be made once the 12-week pre-patent period has passed. Recently, a commercially available coprological antigen ELISA has enabled detection of *F. hepatica* prior to the completion of the pre-patent period, providing earlier diagnosis and increased throughput, although species differentiation is not possible in areas of parasite sympatry. Real-time PCR offers the combined benefits of highly sensitive species differentiation for medium to large sample sizes. However, no molecular diagnostic workflow currently exists for the identification of *Fasciola* spp. in faecal samples.

Methodology/Principal findings

A new molecular diagnostic workflow for the highly-sensitive detection and quantification of *Fasciola* spp. in faecal samples was developed. The technique involves sedimenting and pelleting the samples prior to DNA isolation in order to concentrate the eggs, followed by disruption by bead-beating in a benchtop homogeniser to ensure access to DNA. Although both the new molecular workflow and the traditional sedimentation technique were sensitive and specific, the new molecular workflow enabled faster sample throughput in medium to large epidemiological studies, and provided the additional benefit of speciation. Further, good correlation (R^2 = 0.74–0.76) was observed between the real-time PCR values and the faecal egg count (FEC) using the new molecular workflow for all herds and sampling periods. Finally, no effect of storage in 70% ethanol was detected on sedimentation and DNA isolation outcomes; enabling transport of samples from endemic to non-endemic countries

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without the requirement of a complete cold chain. The commercially-available ELISA displayed poorer sensitivity, even after adjustment of the positive threshold (65–88%), compared to the sensitivity (91–100%) of the new molecular diagnostic workflow.

Conclusions/Significance

Species-specific assays for sensitive detection of *Fasciola* spp. enable ante-mortem diagnosis in both human and animal settings. This includes Southeast Asia where there are potentially many undocumented human cases and where post-mortem examination of production animals can be difficult. The new molecular workflow provides a sensitive and quantitative diagnostic approach for the rapid testing of medium to large sample sizes, potentially superseding the traditional sedimentation and FEC technique and enabling surveillance programs in locations where animal and human health funding is limited.

Author summary

Fasciolosis caused by infection with *F. hepatica*, *F. gigantica* and their hybrid strains is an important health issue in medical and veterinary sciences. The economic impacts of infection and disease on production animals is of particular concern in low-income rural areas of developing countries, including in Southeast Asia where aquatic rice production plus low-input large ruminant husbandry, provides an ideal habitat for parasite proliferation. Here, we describe the development of a robust approach to ante-mortem diagnosis capable of differentiating between the two *Fasciola* spp. and their hybrid. Previously, such differentiation was based on identification of flukes collected post-mortem, because eggs of the two species have overlapping morphology. The major novelty is the demonstration that *Fasciola* spp. eggs, released by adults in the bile ducts, can be effectively and reproducibly broken and the content made available for DNA isolation. Coupled with part of a traditional sedimentation technique, this approach presents a new molecular diagnostic workflow capable of the specific detection of *F. hepatica* DNA in samples with $\leq$10 eggs per gram of faeces using a real-time PCR TaqMan assay. The presented workflow enables differentiation of *F. hepatica* and *F. gigantica* and their hybrid if a duplex TaqMan real-time PCR assay is included. The ability to process medium to large sample sizes *in lieu* of a continuous cold chain will enable further research into the epidemiology, control and public health concerns associated with *Fasciola* spp. infection.

Introduction

Fasciolosis, due to *Fasciola hepatica* and *Fasciola gigantica*, is an important zoonotic production-limiting disease of ruminants [1]. In 2005, an estimated 91 million people across 8 countries were considered at risk of contracting this neglected tropical disease, with children the most likely to become infected [2]. Despite the number of people at risk, fasciolosis is generally considered a neglected disease of humans due to its chronic nature and subsequent underreporting [3]. Livestock and wildlife act as definitive hosts, although it has been demonstrated that humans may also play a participatory role in the spread of the parasite [4]. Human infection typically occurs through the ingestion of freshwater plants such as watercress, although

infection via consumption of metacercariae-contaminated water has also been documented [5].

Infections are traditionally diagnosed via the sedimentation of faecal samples, or more recently through immunological tests such as a commercially available coprological antigen ELISA (coproELISA) [6, 7]. Neither of these methods can differentiate *F. hepatica* from *F. gigantica* or *vice versa* [8]. A high degree of operator error is associated with sedimentation outcomes, with faecal egg count (FEC) results often differing between operators, depending on their level of experience. Species identification by extraction of adult flukes requires post-mortem analysis of the liver, which is not possible in human cases and often of limited availability in ruminants unless conducted during abattoir surveillance. Other non-invasive forms of diagnosis in humans and animals, including serological techniques, are unable to provide species confirmation [7]. DNA isolation and real-time PCR analysis have the potential for a preferred diagnostic solution, offering increased throughput, reproducibility, and higher sensitivity, with the added benefit of species differentiation. Despite this potential, no published method currently exists for the reliable, highly sensitive and specific diagnosis of infection from faecal samples.

The aim of this study was to develop a new molecular diagnostic workflow for real-time PCR detection of *F. hepatica* eggs in ruminant faecal samples and to subsequently compare the results to a traditional sedimentation diagnostic test. The workflow involves an optimised disruption protocol to isolate DNA from *Fasciola* spp. eggs, and a real-time PCR assay that was evaluated for sensitivity and specificity. Additionally, DNA isolation and real-time PCR of a partial and whole pellet from a traditional sedimentation was tested to determine if these additional steps improved the analytical sensitivity of the PCR assay. Finally, the diagnostic sensitivity and specificity of the coproELISA was evaluated in comparison to the traditional sedimentation and newly-developed molecular diagnostic workflow. The positive cut-off threshold was assessed in order to increase the sensitivity and specificity of the ELISA for samples with low egg counts ($\leq$10 eggs per gram, EPG). The end result is a new molecular workflow for the diagnosis of *F. hepatica* that was applied to samples from a cohort of beef cattle with constant *F. hepatica* exposure. Further, the impact of sample storage in 70% ethanol (EtOH) on sedimentation outcomes and DNA isolation and amplification were assessed to determine the feasibility of transporting samples in situations where cold chains may not be readily available. The development of a new molecular diagnostic workflow would enable the highly sensitive detection of *Fasciola* spp. for the quantification of faecal egg load and species identification in regions of parasite sympatry, such as Southeast Asia.

Materials and methods

Faecal samples

***Fasciola hepatica* infected sheep faecal samples.** Faecal samples from Merino sheep (n = 10) positive for *F. hepatica* were collected from a property near Kemps Creek in New South Wales in October 2016 during routine animal handling by a licensed veterinarian. Samples were submitted for parasitological diagnostics (Sydney School of Veterinary Science, The University of Sydney) where they were stored at 4˚C for up to two weeks prior to processing.

***Fasciola hepatica* infected cattle faecal samples.** Faecal samples (n = 31) from Murray Grey beef cattle greater than two years of age suspected to have been exposed to *F. hepatica* infection during a flooding event in 2015 were collected five months apart (October 2016 and February 2017; spring and summer, respectively (S1 Table)). The grazing cattle were located on a property near Newcastle (Herd 1), New South Wales, and submitted for parasitological diagnostics (Sydney School of Veterinary Science, The University of Sydney). No flukicide

treatment had previously occurred on the property. A further 10 samples were submitted for parasitological examination in March 2017 from a cohort of mixed beef cattle (Brangus, Charolais) on a nearby property in Newcastle (Herd 2), New South Wales, with endemic fasciolosis. Herd 2 had been treated with triclabendazole six months prior to sample collection. All samples were collected by a licensed veterinarian during routine animal handling for diagnostic purposes (Sydney School of Veterinary Science, The University of Sydney). All animals from both Herd 1 and 2 had concurrent infections with paramphistome species (Bovine stomach fluke). Samples were stored at 4˚C for up to four weeks prior to processing.

***Fasciola gigantica* infected cattle faecal samples.** Faecal samples (n = 5) positive for *F. gigantica* from local native cattle in Cambodia were collected in February 2017 during routine animal health checks by a licensed veterinarian and stored in 70% EtOH before being shipped to Australia for real-time PCR analysis (Sydney School of Veterinary Science, The University of Sydney).

Sedimentation and faecal egg count

FECs were determined by a standard faecal sedimentation method with minor modifications as follows [9]. Faecal samples (3 g and 6 g for sheep and cattle, respectively) were mixed with distilled water to form a homogenous solution. The solution was hosed with tap water through a 270 μm nylon sieve into a 250 ml conical measuring cylinder, topped with distilled water and allowed to sediment for three minutes. After three minutes the supernatant was aspirated and the sediment poured into a 100 ml conical measuring cylinder, topped with distilled water and allowed to sediment for a further three minutes. Again, the supernatant was aspirated and the remaining sediment poured into a 15 ml centrifuge tube, where it was once more topped with distilled water and allowed to sediment for a final three minutes. The supernatant was aspirated and discarded, leaving 2 ml of sediment which was thoroughly vortexed to ensure homogeneity. To examine presence of fluke eggs, 2 drops of methylene blue (1%) was added to the sediment and examined under an Olympus LG-PS2 stereomicroscope using a 6.5×17×1 cm grid tray at 15× magnification. An additional 20 ml of distilled water was added to the tray to allow ease of counting. Each faecal sample was sedimented and counted in duplicate, resulting in a total of 6 g and 12 g being counted for individual sheep and cattle, respectively. All yellow-brown *Fasciola* spp. eggs were counted.

Triplicate clean faecal samples were spiked with a known number of *F. hepatica* eggs and the percentage lost during the sedimentation process was calculated. The results were in agreement with the original protocol and demonstrated that one third of eggs from the initial sample volume were retained in the sediment after processing [9]. Hence the final number of eggs from individual cattle sedimentations was divided by 2 to obtain EPG (S2 Table). All EPGs are presented as a mean of the two independent sedimentations. All sedimentations were conducted by the same technician to remove any variability in counting. The technician was unaware of the previous results to prevent bias between replicate counts.

Adult *Fasciola* spp. samples

Clean adult *F. hepatica* and *F. gigantica* flukes stored in 70% EtOH from the parasite collection at the Sydney School of Veterinary Science, University of Sydney were used as positive controls. Total genomic DNA from 1/5th of an adult fluke (25 mg, cut using a sterile scalpel blade) from each species was isolated using Isolate II Genomic DNA kit (BioLine, Australia) according to the manufacturer's instructions and eluted in 100 μl of elution buffer (10 mM TrisCl buffer, pH = 8.5). To monitor DNA isolation efficiency and PCR inhibitors 5 μl of DNA Extraction Control 670 (Bioline, Australia) was included and DNA assayed for presence of

extraction control signal on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia) using SensiFAST Probe No-ROX Mix (BioLine, Australia) according to the manufacturer's instructions with expected C_T values of <31. Each DNA isolation batch included a blank sample (ddH_2O) to detect any potential contamination during the extraction process (extraction negative control). Extracted DNA was stored at -20˚C prior to molecular analysis.

Real-time PCR efficiency

A set of genus-specific primers were used to specifically amplify *Fasciola* spp. ITS2 rDNA region [10]. The real-time PCR utilised primers SSCP*Fa*F [S0754] (5′-TTG GTA CTC AGT TGT CAG TGT G-3′) and SSCP*Fa*R [S0755] (5′-AGC ATC AGA CAC ATG ACC AAG-3′) generating 140 bp amplicons [10]. *F. hepatica* species specific TaqMan probe ProFh [S0770] (5'-ACC AGG CAC GTT CCG TCA CTG TCA CTT T-3') and *F. gigantica* specific TaqMan probe ProFg [S0771] (5'-ACC AGG CAC GTT CCG TTA CTG TTA CTT TGT-3') were then implemented [10]. Use of probes removed non-specific background amplification detected using SYBR chemistry and provided species-specific confirmation (S1 Fig). The real-time PCR does not amplify paramphistome egg DNA isolated from cattle faecal samples (Herd 2) with concurrent infections (S2 Fig). The TaqMan probes were labelled with a 5'-FAM, 5'-HEX reporter dye, respectively, and 3'-BHQ1 quencher. The assay with Australian samples was run only with FAM labelled ProFh probe because *F. gigantica* is exotic to Australia. All primers and probes were from Macrogen Ltd. (Seoul, Korea). The real-time PCR reactions used SensiFAST Probe No-ROX Mix (BioLine, Australia) on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia). The volumes of the real-time PCR reactions were made up to 20 μl, including 2 μl of template DNA. The PCR mix included primers and probes at final concentrations of 400 nM and 100 nM, respectively. PCR reactions were initiated at 95˚C for 3 min, followed by 40 cycles of 5 s at 95˚C and 10 s at 60˚C. All runs were performed in duplicate and ddH_2O acted as a negative control. The efficiency, limit of detection and limit of quantification of the real-time PCR was determined via seven serial 10-fold dilutions of the positive control (adult *F. hepatica*, 174.6 ng/μl measured with a NanoDrop Nd-1000 spectrophotometer, Thermo Scientific, Australia), representing a range in concentration of 1.75×10^{1} to 1.75×10^{-5} ng/μl. Results were considered to be positive if both replicates displayed C_T values <36.

Optimisation of sample disruption conditions

Disruption of clean eggs. Clean *F. hepatica* eggs in phosphate buffered saline (pH = 7.4) (PBS) were covered to prevent exposure to light and stored at 4˚C for up to two months prior to processing. Total genetic DNA was isolated from the clean eggs using Isolate Faecal DNA kit (BioLine, Australia), with the following modification to the initial homogenisation step. A total of 2000 eggs in 150 μl of PBS were added to the 2 ml DNA isolation kit homogenisation tube with 750 μl of lysis buffer (Isolate Faecal DNA kit, BioLine) and 5 μl of DNA Extraction Control 670 (Bioline, Australia) and homogenised using a high-speed benchtop homogeniser FastPrep-24 (MP Biomedicals, Australia) under the following conditions; (i) 1 × 40 seconds at 4.0 m/s, (ii) 2 × 40 seconds at 4.0 m/s, (iii) 3 × 40 seconds at 4.0 m/s, (iv) 1 × 40 seconds at 6.0 m/s, (v) 2 × 40 seconds at 6.0 m/s and (vi) 3 × 40 seconds at 6.0 m/s, with 5 minute breaks between each 40 second run, at which time the samples were stored on ice. Samples were disrupted and isolated in duplicate for each of the six treatments (i.–vi.) An aliquot of each sample was examined under a light microscope (Olympus BX41, Australia) at 200 × magnification to ensure no intact eggs remained, before being isolated for DNA amplification.

Disruption of eggs in faecal samples. Total genetic DNA was isolated from 150 mg sheep faecal samples with known concentrations of *F. hepatica* eggs (267 EPG) using Isolate Faecal DNA kit (BioLine, Australia) into 100 μl elution buffer (10 mM TrisCl buffer, pH = 8.5), with the following modification to the initial homogenisation step. Samples were added to the 2 ml DNA isolation kit homogenisation tube with 750 μl of lysis buffer (Isolate Faecal DNA kit, Bio-Line) and 5 μl of DNA Extraction Control 670 (Bioline, Australia) and homogenised using a high-speed benchtop homogeniser FastPrep-24 (MP Biomedicals, Australia) under the following conditions; (iv) 1 × 40 seconds at 6.0 m/s, (v) 2 × 40 seconds at 6.0 m/s and (vi) 3 × 40 seconds at 6.0 m/s with 5 minute breaks between each 40 second run, at which time the samples were stored on ice. All subsequent isolations from cattle samples of unknown *F. hepatica* status (Herd 1 and 2) were disrupted at 6.0 m/s for 40 seconds (disruption condition iv.) on the FastPrep-24 (MP Biomedicals, Australia), after which time they were stored on ice until isolation.

For the isolation of DNA from single *F. hepatica* and *F. gigantica* eggs, cattle faecal samples, (Newcastle, Australia and Takeo, Cambodia, respectively), were sedimented and individual eggs were manually removed (with a 20 μl pipette) and placed into 2 ml DNA isolation kit homogenisation tubes with 750 μl lysis buffer (Isolate Faecal DNA kit, BioLine) and 5 μl of DNA Extraction Control 670 (Bioline, Australia). DNA was isolated from five replicates of individual eggs from each species using disruption condition iv. (6.0 m/s for 40 seconds).

Molecular genotyping of *F. hepatica*, *F. gigantica* and paramphistomes

Three conventional PCR assays were used to confirm adult *Fasciola* spp. (Adult *Fasciola* spp. samples) [11, 12]. The internal transcribed spacers 1 (ITS1) and 2 (ITS2) were amplified using primers ITS1-F [S0762] (TTG CGC TGA TTA CGT CCC TG) and ITS1-R [S0763] (TTG GCT GCG CTC TTC ATC GAC) and ITS2-F [S0764] (TGT GTC GAT GAA GAG CGC AG) and ITS2-R [S0765] (TGG TTA GTT TCT TTT CCT CCG C), yielding 639-bp and 519-520-bp-long fragments, respectively [11]. DNA fragments of a 577-bp-long 28S rDNA sequence were amplified using primers 28F1 [S0756] (ACG TGA TTA CCC GCT GAA CT) and 28R600 [S0757] (CTG AGA AAG TGC ACT GAC AAG) [12]. The primers targeting *Fasciola* spp. ITS2 were also used to amplify DNA from paramphistome eggs collected from cattle faecal samples with concurrent infections (Herd 2) [11].

All PCR amplifications were performed with MyTaq Red Mix (BioLine, Australia) in a total volume of 30 μl. Primers were added at a concentration of 250 nM each. The PCR was run using the following cycling conditions: 95˚C for 15 s, 55˚C for 15 s and 72˚C for 20 s for 35 cycles. All reactions were initiated at 95˚C for 2 min and concluded at 72˚C for 7 min. PCRs were amplified in the Verity PCR cycler (Thermo Fisher Scientific, Australia). Each PCR reaction contained 2 μl of sample DNA. All PCRs were run with negative controls (ddH2O). All PCRs that yielded unambiguous single bands of the expected size were directly and bidirectionally sequenced using amplification primers at Macrogen Ltd. (Seoul, Korea) and assembled and compared to reference sequences for *F. hepatica* and *F. gigantica* (AB207139 and AB207143, respectively) in CLC Main Workbench 6.9.1 (Qiagen, CLC Bio) [11].

Diagnostic application

Three sample preparation methods (Fig 1; workflows red [Method 1], green [Method 2] and blue [Method 3]) were used to compare the diagnostic sensitivity and specificity of the real-time PCR on a naturally infected herd with low FECs (Herd 1). For each animal, DNA from 150 mg (Method 1) pure faeces was isolated using the Isolate Faecal DNA kit (BioLine, Australia) and disruption condition iv. Method 2 consisted of a modification to the sedimentation procedure where the final 2 ml of sediment was vortexed to ensure thorough mixing before

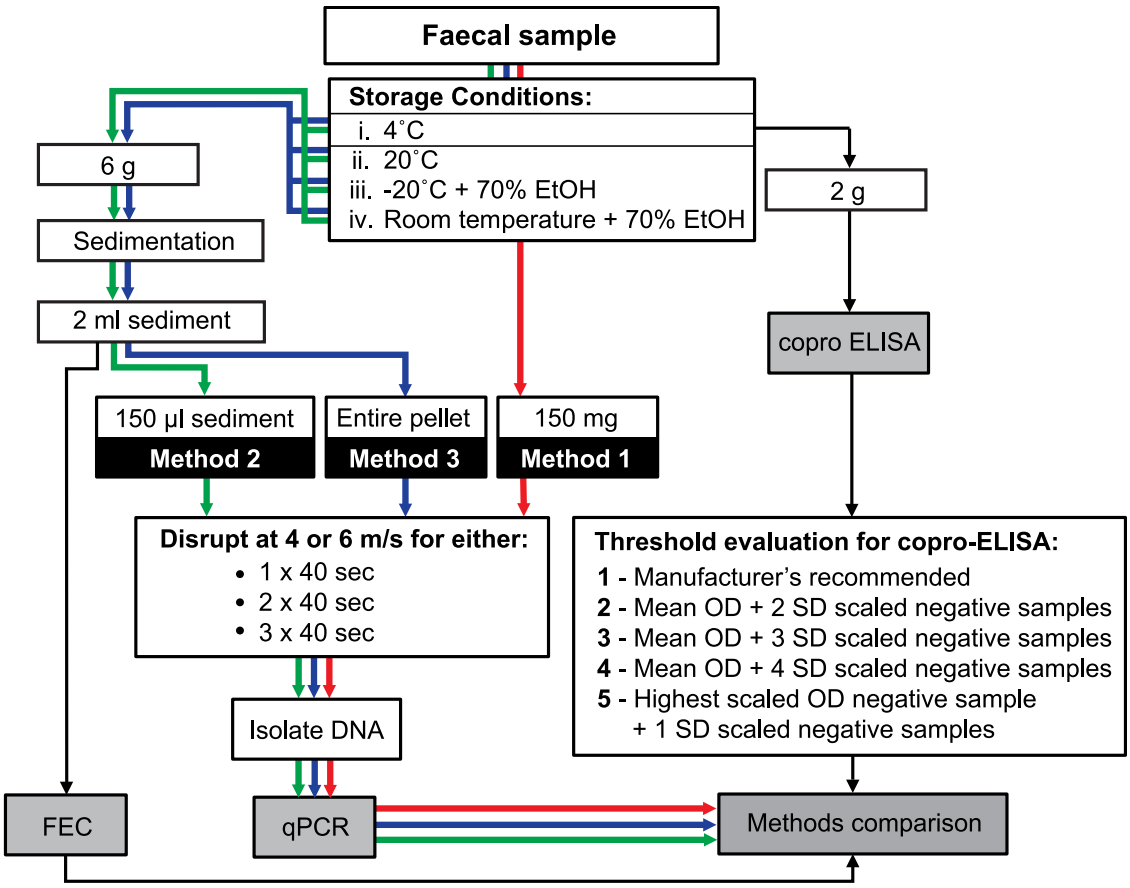

Fig 1. Comparison of three diagnostic methods for the diagnosis of fasciolosis in cattle faecal samples. A traditional sedimentation and faecal egg count (FEC) was performed and the results compared to a coproELISA and real-time PCR. Three methods of sample preparation were compared; isolation of DNA from 150 mg raw faeces (Method 1; red), sedimentation followed by isolation of DNA from 150 µl of the resultant sediment (Method 2; green), and sedimentation followed by isolation of DNA from the entire sediment pellet (Method 3; blue). Egg disruption prior to DNA isolation was assessed using six methods on a benchtop homogeniser using either 4.0 or 6.0 m/s for 1, 2 or 3 rounds of beating for 40 seconds between which samples were stored on ice. The manufacturer-recommended positive threshold of a commercially-available coproELISA was compared to four others suggested in the literature; mean scaled optical density (OD) of negative samples + 2 × standard deviations (SD) of scaled negative samples (Threshold 2) [13], mean scaled OD of negative samples + 3 × SD of scaled negative samples (Threshold 3) [13–15], mean scaled OD of negative samples + 4 × SD of scaled negative samples (Threshold 4) [13], and highest scaled OD of negative samples + 1 SD of negative samples (Threshold 5) [16].

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150 µl (≈150 mg) was removed and DNA isolated using disruption condition iv. A further modification of the sedimentation procedure was employed in Method 3 where the entire 2 ml (≈2 g) of sediment was centrifuged at 2500 g for 10 minutes to form a pellet of concentrated eggs. The entire pellet was manually removed from the 15 ml centrifuge tube using a combination of Pasteur pipettes and fine wooden applicator sticks for DNA isolation using disruption condition iv. The diagnostic sensitivity and specificity of Method 3 was further confirmed on a herd of cattle with constant *F. hepatica* exposure (Herd 2).

The analytical sensitivity of the three sample preparation methods was determined by comparing the morphological FEC from the duplicate traditional sedimentations (henceforth referred to as mEPG) to the FEC calculated according to the standard curve produced by the optimised real-time PCR (henceforth referred to as qEPG).

Impact of storage conditions on sedimentation outcomes and DNA isolation efficiency

To determine the effect of storage conditions on sedimentation and DNA isolation outcomes, 6 g aliquots of clean cattle faecal samples spiked with 2000 *F. hepatica* eggs were stored under the following conditions for one month; i. 4˚C, ii. -20˚C, iii. -20˚C + 70% EtOH, iv. room temperature + 70% EtOH. Samples were spiked and stored in duplicate. After a month, the EtOH was aspirated and samples were placed in an incubator at 37˚C to dry. After drying, each sample was sedimented and counted as described in previously and DNA was isolated as described in the section on diagnostic application (Method 3) under disruption condition iv.

Comparison with a commercially-available coproELISA for detection of *F. hepatica* antigen in faeces

Presence of *F. hepatica* antigen in faecal samples was assayed using 'Monoscreen AgELISA *Fasciola hepatica*' (BIO K 201, Bio-X Diagnostics S.A., Belgium) (coproELISA), an indirect sandwich ELISA kit for the detection of *Fasciola* spp. antigen in cattle and sheep faeces (Fig 1). Cattle faecal samples (Herd 1 and 2) were mixed with the kit dilution buffer 1:1 (2 g + 2 ml) in 12 ml centrifuge tubes and vortexed for 30 seconds until thoroughly mixed followed, by centrifugation for 10 minutes at 2500 g. The supernatant (0.5 ml) was aspirated and stored in labelled microcentrifuge tubes at 4˚C until analysis. There was no effect on positive/negative coproELISA outcomes for samples processed immediately or stored at 4˚C for up to 96 h (S3 Fig; [15]). The coproELISA was performed according to manufacturer instructions with 100 μl of supernatant (prepared as above). Each batch included two positive reference samples as controls. Optical densities (OD) were read at 450 nm using a SpectraMax 250 plate reader (Molecular Devices, LLC., Sunnyvale CA, USA). ODs of each corresponding negative well was subtracted from the individual sample ODs (Net OD). The Scaled OD was calculated by dividing the Net OD of the sample by the Net OD of the positive coproELISA controls. Samples were considered positive for *F. hepatica* antigen if the scaled OD was >0.08 (Monoscreen AgELISA *Fasciola hepatica*, BIO K 201 batch number FASA16B23).

Statistical analysis

As a post-mortem analysis was unable to be performed to confirm the presence or absence of flukes in the liver, the sedimentation technique was considered the gold standard and the diagnostic sensitivity and specificity of the coproELISA and real-time PCR methods were calculated accordingly. Data was analysed in Microsoft Excel (2013) and visualised with GraphPad Prism version 6 (GraphPad Software, USA).

Data accessibility

Nucleotide sequences have been deposited in GenBank (ITS1: MF678648—MF678649; ITS2: MF678650—MF678652; 28S: MF678653—MF678654). The final protocol has been deposited online on protocols.io and can be found at https://dx.doi.org/10.17504/protocols.io.jggcjtw. Raw and supplementary data related to this article have been deposited online on Mendeley Data and can be found under the following DOI's; https://dx.doi.org/10.17632/9zfzv84p8f.2 and https://dx.doi.org/10.17632/4gwjjk47sz.3, respectively.

Table 1. C_T values of 2000 clean *F. hepatica* eggs in phosphate buffered saline (PBS) when subjected to six disruption treatments.

	Treatment						
Replicate	**i.**	**ii.**	**iii.**	**iv.**	**v.**	**vi.**	**SD**
1	16.61	16.23	15.96	16.07	16.14	15.69	0.28
2	16.26	16.04	15.49	16.18	15.65	15.77	0.28

i. 1 × 40 seconds at 4.0 m/s; ii. 2 × 40 seconds at 4.0 m/s; iii. 3 × 40 seconds at 4.0 m/s; iv.1 × 40 seconds at 6.0 m/s; v. 2 × 40 seconds at 6.0 m/s; vi. 3 × 40 seconds at 6.0 m/s.

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Results

Short periods of bead-beating provide consistent *F. hepatica* egg rupture for efficient DNA isolation and amplification

Visual inspection determined that all *F. hepatica* eggs were disrupted after a single round of homogenisation for 40 seconds at 6.0 m/s for both clean eggs and sheep faecal samples. Real-time PCR yielded similar C_T values (15.5–16.6) for all six egg disruption conditions when applied to 2000 clean *F. hepatica* eggs in 150 μl PBS (Table 1). Similarly, when applied to 150 mg of *F. hepatica* infected sheep faecal samples (267 EPG, equivalent to 40 eggs in 150 mg), similar C_T values (21.2–22.0) were observed (Table 2).

The real-time PCR assay was highly efficient (100%, $R^2 = 0.995$) at detecting *F. hepatica* DNA from adult fluke samples. The initial value of the 10-fold serial dilution of *F. hepatica* DNA (1.75×10^1 ng/μl measured with a NanoDrop ND-1000 spectrophotometer, Thermo Scientific, Australia) gave a corresponding C_T value to the 2000 clean eggs in 150 μl PBS (S4 Fig).

The standard curve derived from the serial 10-fold dilution gave intervals of 3.4 C_T values for concentrations of 1.75×10^1 to 1.75×10^{-5} ng/μl (S5 Fig). Henceforth, the *F. hepatica* adult fluke DNA dilution was considered a positive reference and was used to determine qEPG values. The assay routinely detected concentrations of 1.75×10^{-4} mg pure *F. hepatica* DNA (equivalent to a theoretical limit of 2×10^{-2} eggs), demonstrating the limit of quantification and occasionally detected 1.75×10^{-5} mg (equivalent to a theoretical limit of 2×10^{-3} eggs), giving the limit of detection. This theoretical limit was tested in practice through the isolation of DNA from single *F. hepatica* and *F. gigantica* eggs. DNA from five individual eggs from each species was isolated and amplified in duplicate. For both species, 9/10 wells amplified, giving the analytical sensitivity and further demonstrating the value of the bead-beating technique (Table 3).

Table 2. C_T values of *F. hepatica* eggs in sheep faecal samples when subjected to three different disruption treatments.

	Treatment			
Replicate	**iv.**	**v.**	**vi.**	**SD**
1	21.58	21.17	21.74	0.24
2	21.48	21.21	21.96	0.31

iv.1 × 40 seconds at 6.0 m/s; v. 2 × 40 seconds at 6.0 m/s; vi. 3 × 40 seconds at 6.0 m/s.

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Table 3. C_T values from DNA isolation of single *F. hepatica* and *F. gigantica* eggs subjected to 40 seconds of homogenisation at 6.0 m/s (disruption condition iv.).

Sample ID	**1**		**2**		**3**		**4**		**5**		
Replicate	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**Mean Ct (SD)**
F. hepatica	35.16	34.23	34.35	37.67	37.83	-	32.18	32.68	35.29	34.99	34.93 (1.81)
F. gigantica	35.40	35.36	35.16	35.10	36.02	35.72	38.15	-	34.42	34.72	35.51 (1.07)

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A

Sample ID	7	30	32	44	45	48	50	52	53	57	58	63	65	66	67	71	76	78	79	125	132	136	158	189	193	248	249	706	709	710	777	Se (%)	Sp (%)
October (Austral spring)																																	
mEPG*	4	0	4	2	0	9	1	2	4	2	5	6	7	22	2	0	4	0	0	5	1	3	0	0	1	10	5	0	1	0	1	-	-
qPCR - Method 1	N	N	N	Y	N	Y	N	N	Y	N	N	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	27	100
qPCR - Method 2	N	N	N	N	N	N	N	N	N	N	Y	N	N	Y	N	N	N	N	N	Y	N	N	N	N	N	N	Y	N	N	N	N	18	100
qPCR - Method 3	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y	Y	Y	N	N	N	Y	Y	N	N	N	Y	91	89
ELISA - Threshold 1	N	N	N	Y	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	14	100
ELISA - Threshold 2	N	N	Y	Y	N	Y	Y	N	Y	Y	Y	Y	N	Y	Y	Y	Y	N	N	Y	N	N	N	N	N	Y	Y	N	N	N	Y	68	89
ELISA - Threshold 3	N	N	N	Y	N	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N	N	N	N	N	N	N	N	Y	Y	N	N	N	Y	41	100
ELISA - Threshold 4	N	N	N	Y	N	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N	N	N	N	N	N	N	N	Y	Y	N	N	N	Y	41	100
ELISA - Threshold 5	N	N	N	Y	N	Y	N	N	N	N	Y	Y	N	Y	N	N	Y	N	N	N	N	N	N	N	N	Y	Y	N	N	N	Y	41	100
February (Austral summer)																																	
mEPG*	0	0	2	3	0	3	1	1	3	1	4	2	0	4	0	0	4	0	0	1	2	3	0	0	0	6	3	0	1	0	2	-	-
qPCR - Method 3	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	N	Y	N	N	Y	Y	Y	N	N	N	Y	Y	N	Y	N	Y	100	85
ELISA - Threshold 1	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	6	100
ELISA - Threshold 2	N	N	Y	N	N	Y	N	N	Y	N	Y	Y	N	Y	N	N	Y	N	N	Y	N	N	N	N	N	Y	Y	N	Y	N	Y	67	100
ELISA - Threshold 3	N	N	Y	N	N	Y	N	N	N	N	Y	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	Y	Y	N	Y	N	Y	50	100
ELISA - Threshold 4	N	N	Y	N	N	Y	N	N	N	N	Y	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	33	100
ELISA - Threshold 5	N	N	Y	N	N	Y	N	N	Y	N	Y	Y	N	Y	N	N	N	N	N	Y	N	N	N	N	N	Y	Y	N	Y	N	Y	61	100

B

Sample ID	1	2	3	4	5	6	7	8	9	10	Se (%)	Sp (%)
mEPG*	68	10	3	221	1	133	98	0	71	0	-	-
qPCR - Method 3	Y	Y	Y	Y	Y	Y	Y	N	Y	N	100	100
ELISA - Threshold 1	Y	N	N	Y	N	Y	Y	N	Y	N	63	100
ELISA - Threshold 2	Y	Y	N	Y	Y	Y	Y	N	Y	N	88	100
ELISA - Threshold 3	Y	Y	N	Y	N	Y	Y	N	Y	N	75	100
ELISA - Threshold 4	Y	Y	N	Y	N	Y	Y	N	Y	N	75	100
ELISA - Threshold 5	Y	Y	N	Y	N	Y	Y	N	Y	N	75	100

positive (Y) false positive (Y)

negative (N) false negative (N)

* Assumed gold-standard - mEPG (sedimentation)

Fig 2. Comparison of the sensitivity (Se) and specificity (Sp) of three methods for the diagnosis of fasciolosis in cattle faecal samples. (A) FEC results presented as the mean mEPG of two sedimentations in comparison to three methods for real-time PCR analysis and five positive threshold cut-offs for a commercially-available coproELISA for a herd of cattle exposed to fluke two years prior, sampled during the Austral spring and summer. (B) As for A with only one real-time PCR method (Method 3) used on a herd of cattle with endemic fasciolosis. Positive and negative results for each method and threshold are indicated by a Y or N, respectively. Using the FEC data as the assumed gold standard, false negative results are indicated by orange squares, and false positive results are indicated by green squares. Individual animal IDs are presented in the top row for both A and B.

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A new molecular workflow for the sensitive detection of *Fasciola* spp. eggs in faeces

The three methods of sample preparation were compared for detection of *F. hepatica* DNA in faecal samples with low FECs (≤10 EPG). Method 3 proved the most sensitive after disruption at 6.0 m/s for 40 seconds (disruption condition iv.), demonstrating 91–100% diagnostic sensitivity in comparison to the traditional sedimentation technique and FEC in cattle (n = 31) with low FECs (Herd 1) (Fig 2A). Method 3 involved isolating DNA from the entire pellet from a traditional sedimentation, which occasionally exceeded the maximum manufacturer-recommended volume of 150 mg. However, no impact of increased sample volume on DNA isolation and amplification was detected (S3 Table). Method 3 was then used to diagnose *F. hepatica* infection in a cattle herd (n = 10) with constant *F. hepatica* exposure (Herd 2) where 100% diagnostic sensitivity was observed again (Fig 2B). Additionally, Method 3 showed good correlation (0.74–0.76) with FECs for all herds and sampling periods (Fig 3A–3C). In comparison, Methods 1 and 2 show poorer correlation (0.17 and 0.57, respectively) (Fig 3A).

Comparison of the diagnostic sensitivity and specificity of sedimentation (mEPG), real-time PCR (qEPG) and coproELISA diagnostic tests on field samples

More than half (71% and 58%, October 2016 and February 2017, respectively) of Herd 1 (n = 31) was positive for *F. hepatica* by sedimentation on both collection dates (Fig 2A and

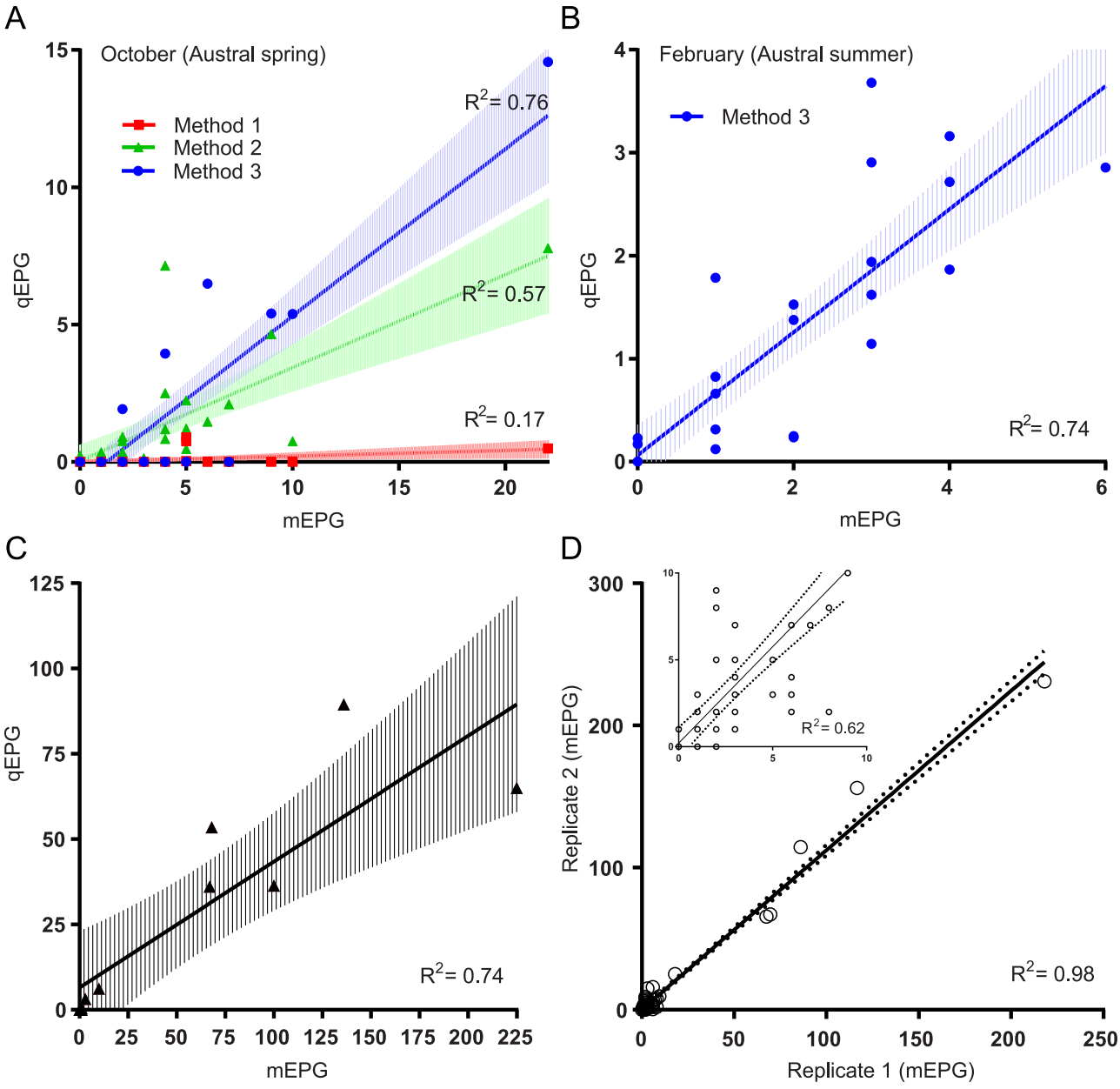

Fig 3. Correlation between faecal egg counts and real-time PCR egg estimates. (A) Real-time PCR egg estimates (qEPG) for three methods of sample preparation for DNA isolation from cattle samples collected during Austral spring (Herd 1) are compared to morphological FEC (mEPG); isolation of DNA from 150 mg raw faeces (Method 1), sedimentation followed by isolation of DNA from 150 μl of the resultant sediment (Method 2), and sedimentation followed by isolation of DNA from the entire sediment pellet (Method 3). (B) As for A using only Method 3 on samples collected during Austral summer (Herd 1). (C) As for B using samples collected from an endemic population (Herd 2). (D) Correlation between duplicate sedimentations (mEPG) for all samples (Herds 1 and 2) across all sampling periods; inset–correlation between duplicate FECs for samples with ≤ 20 EPG.

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Table 4). The average FEC result for Herd 1 during the Austral spring and summer was 5 EPG (±4.55) and 3 EPG (±1.34), respectively (Table 4). For Herd 2, 80% (8/10) of the animals were positive for *F. hepatica* by faecal sedimentation and the average FEC was 61 EPG (±70.25) (Fig 2B and Table 4). A strong positive correlation (0.98) was observed between duplicate FECs (Fig 3D).

Table 4. *F. hepatica* faecal egg counts (FEC) from two cattle herds located in Newcastle, New South Wales.

	Herd 1		Herd 2
	October 2016	February 2017	March 2017
Total Samples	31	31	10
Total Positive (%)	22 (71)	18 (58)	8 (80)
Mean EPG (±SD)	4.59 (±4.55)	2.56 (±1.34)	60.50 (±70.25)

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A commercially available coproELISA was used to diagnose *F. hepatica* infection in both Herd 1 and 2 across all sampling periods and the diagnostic sensitivity and specificity at five different thresholds was calculated (Fig 2). Threshold 2 proved to be the most sensitive cut-off and was calculated by averaging the scaled OD and adding two standard deviations of the known negative samples [13]. Using this cut-off the diagnostic sensitivity ranged from 65–88% for both herds over all sampling periods. In comparison, the manufacturer's recommended cut-off yielded a larger range in diagnostic sensitivity of 6–63% for both herds over the two sampling periods (Fig 2).

When using the new molecular workflow (disruption condition iv. and sample preparation Method 3) as the gold standard of *Fasciola* spp. diagnosis, the diagnostic sensitivity and specificity of the traditional sedimentation technique is 90–100% and 80–100%, respectively (S4C Table). In comparison, the diagnostic sensitivity and specificity of the coproELISA at Threshold 2 is 60–88% and 100%, respectively (S4D Table).

Differentiation of *F. hepatica* from *F. gigantica* using DNA isolated from eggs in faeces

The DNA isolated using disruption protocol iv. was suitable for the differentiation between *F. hepatica* and *F. gigantica* for both conventional and real-time PCR. *F. hepatica* DNA isolated from cattle faecal samples (Herd 1) was used to successfully amplify and sequence a 28S rDNA gene fragment 100% matching our reference 28S rDNA (MF678654). The 28S rDNA gene fragment is 99.5% identical (3 nt differences across 577 nt) between *F. hepatica* (MF678654) and *F. gigantica* (MF678653). Primers targeting ITS1 and ITS2 are not *Fasciola* spp. specific i.e. they also amplify paramphistome DNA, therefore were not used in genotyping PCRs with DNA isolated from faecal samples.

Storage condition has no effect on DNA isolation outcomes enabling transport of samples in the absence of a complete cold chain

Storage conditions had no effect on sedimentation, DNA isolation and amplification outcomes (Table 5). Regardless of the storage condition applied, sedimentation results remained consistent across all replicates and DNA amplification remained unaffected (S5 Table).

Table 5. C_T values of samples subjected to different storage conditions prior to sedimentation and DNA isolation.

	Treatment				
Replicate	**4˚C**	**-20˚C**	**-20˚C + 70% EtOH**	**Room Temperature + 70% EtOH**	**SD**
1	22.42	22.13	21.35	22.32	0.42
2	22.85	21.77	21.67	21.85	0.26

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Discussion

A new molecular workflow was developed in response to the need for a specific diagnostic tool for *Fasciola* spp. in faecal samples [17–19]. The technique enabled medium to large sample throughput with high sensitivity in order to detect changes in faecal *Fasciola* spp. egg load, with the added benefit of parasite speciation (i.e. *F. hepatica* vs *F. gigantica*). In the present study, the extraction of DNA from *F. hepatica* eggs through the use of a bead-beating approach resulted in consistent DNA isolation. The bead-beating approach (iv.) was applied to raw faecal samples with low *F. hepatica* EPG, but demonstrated decreased diagnostic sensitivity. To improve the diagnostic sensitivity, a *Fasciola*-egg concentration technique through egg sedimentation (Method 3) was combined with the bead-beating approach prior to DNA isolation. The new molecular workflow was highly sensitive (91–100%) with the real-time PCR results showing good correlation with faecal egg counts (0.74–0.76), enabling robust quantitative detection of *Fasciola* species-specific eggs in faeces.

The hard shell of *Fasciola* spp. eggs must first be disrupted to ensure access to the inner contents for DNA isolation and subsequent amplification. Although mechanical disruption prior to DNA extraction has been previously employed for the isolation of *Fasciola* spp. DNA from faeces, demonstration of the efficiency of mechanical disruption is lacking [20–22]. Our study shows consistent mechanical rupture of *F. hepatica* eggs across a wide range of settings on a high-speed benchtop homogeniser. Our results of mechanical egg disruption are consistent with findings for other parasitic species with notoriously robust eggs, such as *Trichuris trichiura* and *Echinococcus multilocularis* [23–24]. The DNA isolation protocol successfully isolated DNA from single *F. hepatica* and *F. gigantica* eggs using a single short 40s period of bead-beating (disruption condition iv.). It has previously been shown that isolation of DNA from a single *Fasciola* spp. egg was possible after vortexing with glass beads for 30 minutes [21]. In a diagnostic laboratory setting, a rapid sample preparation and DNA isolation approach is paramount to take advantage of fast real-time PCR assays to deliver reproducible and accurate diagnostic results. The wide range of settings capable of disrupting *Fasciola*-eggs (disruption conditions i.–iv.) allowed for the selection of a bead-beating protocol that best aligns with other faecal sample molecular diagnostic procedures. As our laboratory utilises a standard protocol that includes 40s at speed of 6.0 m/s using FastPrep-24 (MP Biomedicals, Australia), this was incorporated into the new molecular workflow for *Fasciola* spp. DNA diagnostics. With the demonstration of a wide range of suitable settings able to disrupt *Fasciola*-eggs for DNA isolation, adaptation of our workflow for other laboratories will be appropriate.

Chronic *Fasciola* spp. infection in animals and humans give variable egg outputs, therefore we optimised our workflow to maximise the analytical sensitivity to <10 EPG [9,25–27]. Our initial success with clean eggs demonstrated consistent DNA isolation, although applying this approach to naturally-infected faecal samples with low EPGs (<10 EPG) proved challenging. When using the manufacturer-recommended maximum volume of raw faecal material (150 mg) the theoretical sensitivity is 6.67 EPG, assuming that each 150 mg of raw faecal material contains 1 *Fasciola* spp. egg (Method 1). This is in agreement with other work [28] reporting the analytical sensitivity of one *F. gigantica* egg in 100 mg faeces, equivalent to 10 EPG. Thus, Method 1 is of limited diagnostic use in chronically infected cattle that frequently report low egg numbers (≤10EPG) [25,27]. To improve the approach, a concentration step was included which employed the traditional sedimentation technique for trematode eggs [9] without the microscopic observation and counting (Method 3). A previously-described alternative method for egg concentration [21] used laborious washing and sieving procedures, including overnight refrigeration of a faecal suspension, making it inappropriate for application in diagnostics [29]. Our new molecular workflow incorporates a *Fasciola*-egg concentration procedure prior

to the optimised *Fasciola*-egg disruption protocol, leading to highly successful results of 91–100% diagnostic sensitivity in a pilot study in cattle with ≤10 EPG.

To ensure that the diagnostic sensitivity of the optimised workflow was consistent across a range of faecal egg loads we tested our approach on ten samples from an endemically infected herd that had received a triclabendazole oral drench six months prior to sampling. Despite the smaller sample size, these samples were considered suitable for proof of principal of the optimised workflow due to their considerably larger EPG range (max. 221EPG, mean 61EPG) and our previous success across two time points in a herd with low EPGs. To our knowledge only two other studies [20,22] report results testing the capability of molecular diagnostic tools for the identification of *Fasciola* spp. infection in individual naturally infected animals. In contrast, the diagnostic capacity of our optimised workflow remained high [20]. The clear benefits of the concentration of eggs in samples prior to isolation address the diagnostic sensitivity limitations previously highlighted [20]. No details were provided in other work [22] regarding the sensitivity of the diagnostic approach on naturally infected samples. However, we maintain that our consistent results across different groups of naturally infected animals demonstrate the robustness of our approach to sample preparation for DNA isolation, regardless of the molecular tools employed. Hence the clear benefits of the new molecular workflow addresses the needs of the animal and human health industry in regards to increasing the analytical and diagnostic sensitivity of *Fasciola* spp. molecular diagnosis.

New antigen detection techniques for the diagnosis of *Fasciola* spp. infection (coproELISA) have been used to address the limitations of the traditional sedimentation and FEC approach by detecting infection prior to the completion of the pre-patent period [30]. We compared the diagnostic sensitivity and specificity of all three methods, including our new molecular workflow, by additionally diagnosing all cattle samples with the commercially-available coproELISA. Several studies have reported a decreased sensitivity of the coproELISA since commercialisation when diagnosing samples containing ≤10 EPG and using the manufacturers recommended positive threshold [15–16]. This is despite reporting detection limits of 0.6 ng/ml (cattle) and 0.3 ng/ml (sheep) of *Fasciola* spp. antigen, corresponding to a sensitivity of 100% for cattle harbouring 2 or more flukes, or sheep harbouring 1 fluke, during development [6]. In our study, samples <10 EPG were in agreement with previous reports, and in response we re-evaluated the positive threshold by employing several previously-described methods [13–16]. This re-evaluation increased the diagnostic sensitivity of the coproELISA, particularly in the recently infected herd (Herd 2). However, the application of arbitrary statistical methods to increase the sensitivity of the assay is problematic, as these methods are unlikely to be applicable across each new population being tested [13]. Hence, a new positive threshold must be calculated for each new batch number and species being diagnosed, resulting in additional costs and an unnecessary waste of time, particularly where large samples sizes are involved. Further, despite the added benefits of earlier diagnosis, the lack of ability to speciate still remains in regions such as Southeast Asia with both *Fasciola* spp. present. While still being limited by the pre-patent period, the new molecular workflow for the detection of *Fasciola* spp. in faecal samples maintains the larger throughput associated with the coproELISA at a similar cost, whilst providing the added benefit of species differentiation.

The new molecular workflow provides a simple step-wise process for the preparation of faecal samples enabling medium-high throughput for the diagnosis of Fasciolosis. However, the application of this approach is of limited use in locations where the diagnostic capacity may be restricted or if the necessary laboratory equipment is lacking. The ability to preserve samples in 70% EtOH and transport them to areas with increased diagnostic capacity is vital, particularly when conducting epidemiological studies on Fasciolosis in remote and rural areas such as Southeast Asia. The opportunity of sample preservation for transport has been demonstrated

previously, particularly when working in areas lacking a continuous cold chain [23,31]. Our results were in agreement, demonstrating that regardless of the storage conditions we applied, there was no effect on sedimentation and DNA isolation.

The advancement of molecular tools for the differentiation of *Fasciola* spp. have greatly added to our understanding of their ecology, epidemiology and zoonotic potential [19]. However, no tool currently exists for the identification of the hybrids between *F. hepatica* and *F. gigantica* in the field. This is especially important in areas where *F. hepatica*, *F. gigantica* and their hybrids exist in sympatry, such as Southeast Asia, where the zoonotic potential of the hybrid forms are largely unknown [19, 32]. The inclusion of TaqMan probes in our new molecular diagnostic workflow enables the identification of either infection with a hybrid, or a mixed infection with both *F. hepatica* and *F. gigantica* within a single animal [10]. However, due to the triploid nature of the hybrids, the differentiation between these two scenarios would require the additional isolation of DNA from single eggs [33]. Our new molecular diagnostic workflow provides this capability, as demonstrated by the repeated successful isolation of DNA from single eggs of both *F. hepatica* and *F. gigantica*, adding an additional tool to our diagnostic arsenal.

In conclusion, we present a robust approach for the ante-mortem diagnosis of *Fasciola* spp. infection using faecal samples. The presented workflow is able to differentiate between *F. hepatica* and *F. gigantica* species, while also providing a flexible methodology capable of being adapted for use in existing diagnostic laboratory workflows. Although the method maintains the requirement for the completion of the pre-patent period, the additional benefits of fluke species differentiation and increased sample throughput provide clear benefits over the traditional sedimentation and FEC approach. The high diagnostic sensitivity and ability to store samples in 70% EtOH make this approach suitable for use in surveillance programs and epidemiological studies in areas where access to a complete cold chain is lacking or where laboratory capacity is limited.

Supporting information

S1 Table. Newcastle climate data.
(XLSX)

S2 Table. Percentage of eggs lost during sedimentation.
(XLSX)

S3 Table. Impact of faecal sample volume on DNA isolation.
(XLSX)

S4 Table. Diagnostic sensitivity and specificity.
(XLSX)

S5 Table. Impact of storage conditions on sedimentation outcomes and DNA isolation.
(XLSX)

S1 Fig. Removal of non-specific amplification with probes.
(PDF)

S2 Fig. No amplification of paramphistome DNA.
(PDF)

S3 Fig. No effect of storage time on positive/negative coproELISA outcomes.
(PDF)

S4 Fig. Egg and fluke corresponding values.
(PDF)

S5 Fig. Standard curve.
(PDF)

S6 Fig. STARD checklist.
(PDF)

S7 Fig. STARD flowchart.
(PDF)

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Author Contributions

Conceptualization: Nichola Eliza Davies Calvani, Jan Šlapeta.

Data curation: Nichola Eliza Davies Calvani, Jan Šlapeta.

Formal analysis: Nichola Eliza Davies Calvani.

Funding acquisition: Peter Andrew Windsor, Russell David Bush, Jan Šlapeta.

Investigation: Nichola Eliza Davies Calvani, Peter Andrew Windsor, Russell David Bush, Jan Šlapeta.

Methodology: Nichola Eliza Davies Calvani.

Project administration: Jan Šlapeta.

Resources: Jan Šlapeta.

Supervision: Peter Andrew Windsor, Russell David Bush, Jan Šlapeta.

Validation: Nichola Eliza Davies Calvani.

Writing – original draft: Nichola Eliza Davies Calvani.

Writing – review & editing: Nichola Eliza Davies Calvani, Peter Andrew Windsor, Russell David Bush, Jan Šlapeta.

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Chapter 3

Comparison of early detection of *Fasciola hepatica* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Nichola Calvani in the preparation and submission of the following manuscript:

> Calvani, N.E.D., George, S.D., Windsor, P.A., Bush, R.D. and Šlapeta, J. (2018). Comparison of early detection of *Fasciola* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation. Veterinary Parasitology 251: 85-89.

Contribution to the manuscript of all authors is as follows:

Task	**Contributing Co-authors**
Conceptualisation	NEDC, JŠ
Data collection and curation	NEDC, SDG, JŠ
Formal analysis	NEDC
Funding acquisition	PAW, RDB, JŠ
Investigation	NEDC, SDG, PAW, RDB, JŠ
Methodology	NEDC, SDG
Supervision, discussion and consultation	SDG, PAW, RDB, JŠ
Preparation of figures used in the manuscript	NEDC, JŠ
Writing of the original draft	NEDC
Critical review and editing of the manuscript	NEDC, SDG, PAW, RDB, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct. I have sighted email or other correspondences from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Kind regards,

Assoc. Prof. Russell Bush
14th January, 2020

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Research paper

Comparison of early detection of *Fasciola hepatica* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation

Nichola Eliza Davies Calvani[a,b], Sarah Deanna George[c], Peter Andrew Windsor[b], Russell David Bush[b], Jan Šlapeta[a,*]

[a] Laboratory of Veterinary Parasitology, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales, Australia
[b] Mekong Livestock Research Group, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales, Australia
[c] Elanco Australasia Pty Limited, Yarrandoo R&D Centre, 245 Western Road, Kemps Creek, New South Wales, Australia

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ABSTRACT

Fasciolosis due to infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a significant global cause of livestock production loss. Infection is commonly diagnosed by a labour-intensive sedimentation and faecal egg count (FEC), which has limited throughput and is only applicable after completion of the 8–12 week pre-patent period (PPP). A commercially-available ELISA for the detection of coprological antigen (coproELISA) enables detection prior to the completion of the PPP and is suitable for diagnosis of larger sample sizes, although the sensitivity reported under experimental infection settings can be difficult to replicate in the field, particularly in cattle. A recently-published real-time PCR workflow for the sensitive detection of *Fasciola* spp. DNA in faecal samples provides increased sample throughput, although the point at which this technique is first able to diagnose infection remains unknown. Other tools for the molecular diagnosis of fasciolosis, such as conventional PCR and loop-mediated isothermal amplification (LAMP), have been shown to detect *F. hepatica* DNA as early as 1 week post infection (WPI). In this study, faecal samples were collected weekly from 10 experimentally-infected Merino lambs and subjected to diagnosis via traditional sedimentation, coproELISA and real-time PCR. Samples were first considered positive at 6–8 WPI by coproELISA, real-time PCR and sedimentation, respectively. At 9 WPI 100% of samples were positive by all three methods. To evaluate the capacity of the real-time PCR approach to detect infection prior to completion of the PPP, two methods of sample preparation were compared at 2 WPI: (i) 150 mg raw faecal samples and (ii) 3 g faecal starting volume prior to sedimentation and pelleting. Neither method of sample preparation yielded positive results at 2 WPI suggesting that DNA amplification by real-time PCR is associated with faecal egg load.

1. Introduction

Fasciolosis caused by infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a zoonotic parasitic disease of global importance (Torgerson and Macpherson, 2011). Up to 91 million people are considered at risk of infection and production losses are estimated to exceed US$2 billion/year (Keiser and Utzinger, 2005; McManus and Dalton, 2006). The parasite has an indirect life cycle, and the pre-patent period (PPP) is generally considered to take 8–12 weeks (Andrews, 1999; Brunsdon, 1967). During this time animal production may be affected due to the migration of immature stages through the liver, causing hepatitis, without externally detectable life cycle stages (Andrews, 1999). Associated production losses include decreased weight gain, anaemia, liver condemnation, reduced reproductive performance and, importantly in the case of acute fasciolosis, the potential for significant increases in animal mortality, often with minimal warning (Andrews, 1999).

Traditionally, ante mortem diagnosis has been performed by faecal egg counts (FEC) utilising either sedimentation or flotation techniques (Happich and Boray, 1969). These techniques have limited sensitivity for animals infected with liver flukes and are only of use after the PPP has passed (Happich and Boray, 1969). Recently, a commercial ELISA for the detection of fluke antigen in faeces (coproELISA) has enabled detection prior to the completion of the PPP (Mezo et al., 2004). However, issues have been identified regarding the decreased sensitivity of this approach in lighter infections (< 10 eggs per gram, EPG), particularly in cattle (George et al., 2017b; Gordon et al., 2012; Kajugu et al., 2015; Martinez-Sernandez et al., 2016). A serological ELISA is commercially available and in naïve animals provides the earliest indication of infection (Mezo et al., 2007). However, a prolonged

* Corresponding author.
E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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antibody response prevents this approach from detecting reinfection, limiting its application to surveillance of exposure (Mezo et al., 2007). A molecular approach utilising loop-mediated isothermal amplification (LAMP) has suggested that DNA may be detected in faeces from as early as one week post infection (WPI) in experimental studies (Martinez-Valladares and Rojo-Vazquez, 2016). Similarly, a real-time PCR-based workflow was recently published demonstrating high sensitivity in cattle faecal samples with low FECs (< 10 EPG) (Calvani et al., 2017). This approach has never been tested in an experimental infection setting and thus the point at which this technique is first able to detect *Fasciola* spp. DNA in faecal samples remains unknown (Calvani et al., 2017).

The present study aims to evaluate the earliest point of detection of *F. hepatica* in faecal samples in experimentally infected sheep using a recently described real-time PCR-based molecular workflow (for the detection of DNA from eggs), when compared to a traditional sedimentation (for the detection of eggs) and the commercially-available coproELISA (for the detection of coproantigen released by metabolically active flukes). To determine the presence of *F. hepatica* DNA in faecal samples prior to egg shedding we tested raw and sedimented faecal samples at two weeks post infection.

2. Materials and methods

2.1. Experimental infection

All animal experimentation was conducted at Yarrandoo R&D Centre (NSW, Australia). Ten commercially-sourced 10 month old Merino lambs were confirmed to be negative for *F. hepatica* prior to infection by sedimentation 12 weeks post removal from pasture. Animals were housed in group conditions in an indoor facility and fed a lucerne hay/oaten hay/straw/oats chaff mix, supplemented with a lucerne-based concentrate pellet. Water was provided *ad libitum* via the town supply system. Sheep were inspected at least daily for the duration of the sampling period. Animal ethics approval was provided by the Elanco Australasia Pty Limited Animal Ethics Committee (approval ELA170004).

Each animal was infected per os with 250 *F. hepatica* metacercariae generated at the Yarrandoo R&D Centre (New South Wales, Australia) for the purpose of strain maintenance. Weekly faecal samples were collected per rectum from 2 to 11 WPI. Four distinct Australian isolates of *F. hepatica* were used in order to maintain experimental strains; 'Oberon' (n = 2), 'Numbugga' (n = 2), 'Palmers Oaky' (n = 5) and 'Bombala' (n = 1). The 'Oberon' strain was isolated by the NSW Department of Primary Industries in 1999 and the remaining strains were isolated at the Yarrandoo R&D Centre (Fairweather, 2011; George et al., 2017a). The 'Numbugga' strain was obtained from naturally infected goats near Bega NSW and the 'Palmers Oaky' strain was obtained from sheep grazing near Oberon NSW, both in 2014. The 'Bombala' strain was obtained from sheep grazing the Monaro region of NSW in 2016.

2.2. Sedimentation and faecal egg count

Eggs per gram of faeces (EPG) were determined by a standard sedimentation method with minor modifications as described in Calvani et al. (2017) (Happich and Boray, 1969). Briefly, after initial filtering through 270 um nylon mesh, the modification consisted of three rounds of sedimentation, each three minutes in length, in successively smaller volumes of distilled water (250, 100 and 15 ml). The additional rounds of sedimentation served to remove vegetable matter and did not alter the percentage of eggs retained from the original method (Calvani et al., 2017; Happich and Boray, 1969).

Faecal samples were sedimented and counted in duplicate from 6 to 9 WPI and then once from 10 to 11 WPI. For each replication, 3 g of faeces was used. Duplicate sample EPGs are reported as the mean from two standard sedimentations. Duplicate counts during 6–9 WPI were employed to increase the sample volume to 6 g (2 × 3 g) in order to increase the sensitivity for samples with low counts (< 10 EPG). Samples were counted in duplicate backwards from 9 WPI until a minimum of two time points were negative for each animal. All morphological counts based on microscopic examination are henceforth referred to as mEPG.

2.3. DNA isolation and Fasciola hepatica real-time PCR

Faecal samples from 2 and 6–11 WPI were prepared and DNA isolated according to a previously published molecular diagnostic workflow available online at https://dx.doi.org/10.17504/protocols.io.jggcjtw (Calvani et al., 2017). Briefly, after initial sedimentation and egg-counting, the DNA in the resultant sediment was isolated using Isolate Fecal DNA kit (Bioline, Australia) following the manufacturer's recommendations. Samples were added to the 2 ml DNA isolation kit homogenisation tube with 750 µl of lysis buffer and homogenised in a high speed benchtop homogeniser at 6.0 m/s for 40 s (FastPrep-24, MP Biomedicals, Australia). For the 2 WPI samples, DNA was additionally isolated from 150 mg of raw faeces, homogenised as above and following the manufacturer's recommendations (Bioline, Australia). DNA was eluted into 100 ul elution buffer (10 mM TrisCl buffer, pH = 8.5) and stored at −20 °C prior to amplification. To monitor DNA isolation efficiency and absence of PCR inhibition, 5 ul of DNA Extraction Control 670 (Bioline, Australia) was included and samples were assayed according to manufacturer's instructions.

A TaqMan real-time PCR assay targeting *F. hepatica* ITS2 rDNA was utilised (oligonucleotides SSCP*Fa*F [S0754]/SSCP*Fa*R [S0755] and probe ProFh [S0770] FAM-BHQ1) and run in duplicate (Alasaad et al., 2011). A 10-fold dilution of the positive control of *F. hepatica* DNA served for quantification of egg estimate in EPG as previously described (Calvani et al., 2017). The EPG based on real-time PCR is referred to as qEPG.

All real-time PCR reactions were run on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia) using SsoAdvanced Universal Probes Supermix (BioRad, Australia) according to the manufacturer's instructions and cycling condition described previously (Calvani et al., 2017). Results were considered to be positive if both replicates displayed C_T values < 36. Each batch of DNA isolation was isolated with a blank sample (ddH_2O) to detect contamination that may have occurred during the extraction process. Extraction Control samples with C_T values < 31 were considered not inhibited.

2.4. coproELISA

Faecal samples from 4 to 11 WPI were tested using a commercially available ELISA for the detection of *Fasciola* spp. coprological antigen in faeces (coproELISA) (BIOK 201, Bio-X Diagnostics S.A., Belgium, batch number FASA17A20). Briefly, samples were thoroughly mixed with the kit dilution buffer (0.5 g + 2 ml) in 15 ml centrifuge tubes and allowed to sit overnight at 4C to increase optical density (OD) readings of positive samples as recommended by Brockwell et al. (2013). Optical densities were read at 450 nm using a SpectraMax 250 plate reader (Molecular Devices, LLC., Sunnyvale CA, USA). The OD of each corresponding negative well was subtracted from the individual sample ODs (Net OD). The Scaled OD was calculated by dividing the Net OD of the sample by the Net OD of the positive coproELISA controls. Samples were considered positive for *F. hepatica* antigen if the scaled OD was > 0.08.

3. Statistical analysis and data accessibility

Data was analysed in Microsoft Excel (2013) and visualized using GraphPad Prism version 6 (GraphPad Software, USA). Positive and

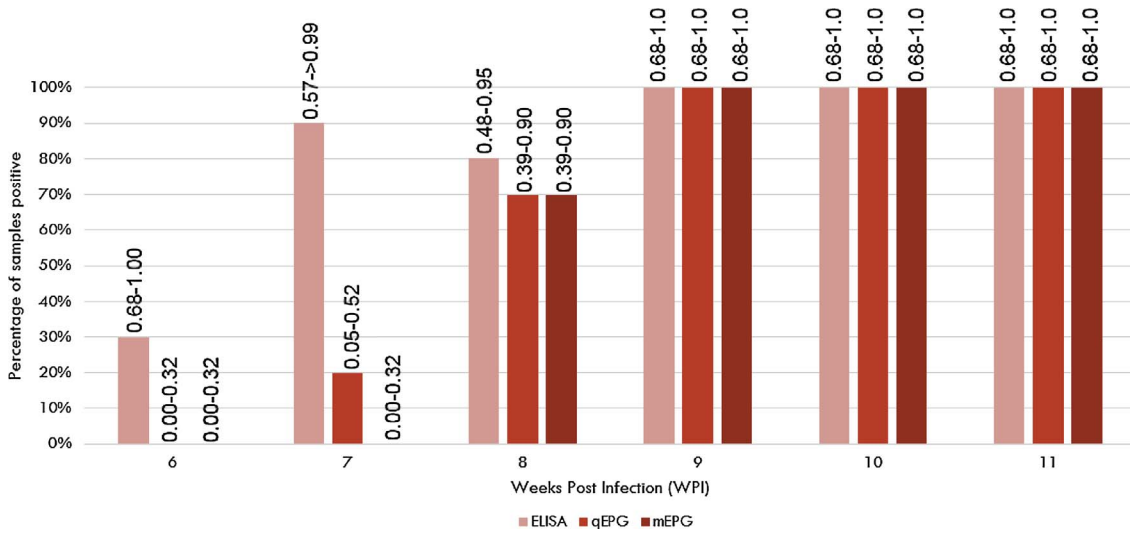

Fig. 1. Percentage of sheep faecal samples per week positive for *F. hepatica* from 6 to 11 WPI using three different detection methods; coproELISA, real-time PCR (qEPG, a molecular estimate of FEC) and a traditional sedimentation (mEPG, morphological FEC results), with 95% confidence intervals listed above columns of each weekly result.

negative test results from each week were converted to binary values (1 = test positive, 0 = test negative) and significance was calculated via ordinary one-way ANOVA in GraphPad Prism version 6 (GraphPad Software, USA). The sedimentation technique was considered the gold standard in lieu of post-mortem examination due to the previously-reported high sensitivity of dual sedimentations for samples with low faecal egg loads (< 10 EPG) (Calvani et al., 2017). Raw data is available under the following DOI: https://http://dx.doi.org/10.17632/hsrngjtckj.1.

4. Results

All animals were successfully infected with their respective *F. hepatica* strains as determined by positive sedimentation, real-time PCR and coproELISA results at 9 WPI (Fig. 1). Result remained positive for all animals until 11 WPI, at which time sampling ceased. No differences in FEC, antigen detection or DNA amplification between strains was observed (data not shown).

Animals were first considered FEC positive at 8 WPI (7/10, 70%), with a mean FEC of 6 EPG (Fig. 2). FECs increased through week 9–11 with mean counts of 71, 236 and 586 EPG, respectively. Sheep 1 and sheep 10 (2/10 sheep, 20%) had FECs < 10 EPG at 9 WPI and gave the lowest FEC for the duration of observation (Fig. 2).

Animals were first considered positive at 6 WPI according to the coproELISA. Positive coproELISA results were seen in 30% of sheep at 6 WPI (95% CI = 0.10–0.61), 90% of sheep at 7 and 8 WPI (95% CI = 0.57 ≥ 0.99), and 100% of sheep at 9 WPI (95% CI = 0.68–1.00) (Fig. 2).

Animals were first considered positive at 7 WPI according to the real-time PCR. DNA was detected in 20% of faecal samples by real-time PCR at 7 WPI (95% CI = 0.05–0.52), 80% of sheep at 8 WPI (95% CI = 0.48–0.95) and 100% of sheep at 9 WPI (95% CI = 0.68–1.00) (Fig. 2). Morphological FEC (mEPG) and the real-time PCR EPG estimate (qEPG) were highly correlated (R^2 = 0.94, Fig. 3).

Outcomes for the three different tests, coproELISA, real-time PCR and FEC were considered significantly different at weeks 6 and 7 (P-

	ID	Sheep 1			Sheep 2			Sheep 3			Sheep 4			Sheep 5		
		ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG
Week	4	0%	n/a	n/a	0%	n/a	n/a	1%	n/a	n/a	0%	n/a	n/a	1%	n/a	n/a
	5	2%	n/a	n/a	1%	n/a	n/a	0%	n/a	n/a	0%	n/a	n/a	2%	n/a	n/a
	6	3%	0	0	5%	0	0	36%	0	0	24%	0	0	3%	0	0
	7	30%	1	0	12%	0	0	62%	0	0	36%	1	0	24%	0	0
	8	30%	0	0	22%	1	1	26%	65	30	44%	2	1	23%	40	17
	9	104%	5	1	68%	126	34	128%	721	227	139%	608	82	25%	366	99
	10	134%	71	13	104%	1006	70	123%	3094	886	151%	3129	494	64%	462	317
	11	150%	482	127	100%	2635	474	150%	5029	1043	147%	3461	766	71%	4127	847

	ID	Sheep 6			Sheep 7			Sheep 8			Sheep 9			Sheep 10		
		ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG	ELISA	qEPG	mEPG
Week	4	0%	n/a	n/a	2%	n/a	n/a	3%	n/a	n/a	0%	n/a	n/a	1%	n/a	n/a
	5	2%	n/a	n/a	1%	n/a	n/a	3%	n/a	n/a	2%	n/a	n/a	3%	n/a	n/a
	6	3%	0	0	3%	0	0	6%	0	0	12%	0	0	2%	0	0
	7	1%	0	0	18%	0	0	66%	0	0	46%	0	0	37%	0	0
	8	7%	35	5	5%	0	0	46%	0	1	79%	27	2	61%	2	0
	9	19%	231	72	100%	85	17	120%	149	50	57%	539	128	44%	12	4
	10	57%	618	201	124%	337	56	92%	442	90	97%	472	169	121%	308	60
	11	116%	2981	545	152%	2527	609	125%	1820	414	74%	2392	631	158%	2298	406

Fig. 2. Heatmap comparison of three approaches for the detection of *F. hepatica* in experimentally infected sheep; coproELISA, real-time PCR (presented as qEPG, a molecular estimate of FEC) and a traditional sedimentation (presented as mEPG, morphological FEC results). The positive threshold for the coproELISA was set at 8% as per the manufacturer's instructions. Both the mEPG and qEPG were considered positive at 1EPG.

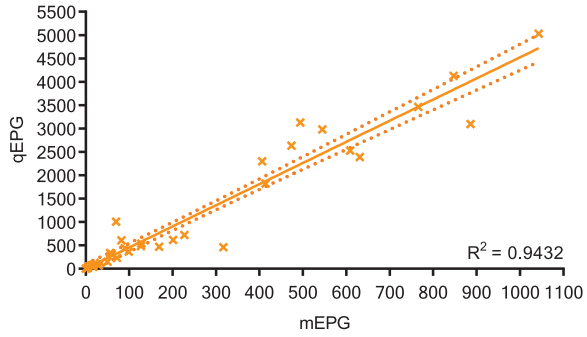

Fig. 3. Correlation between morphological FEC (mEPG) and molecular FEC estimates (qEPG) in experimentally infected sheep.

value = 0.0336 and < 0.0001, respectively), but not at week 8 (P-value = 0.8570).

To evaluate the presence of "free DNA" in the early stages of infection two methods of DNA isolation were applied to the 2 WPI faecal samples. DNA was isolated from 150 mg raw faeces or from the resultant sediment from a traditional sedimentation. Neither method of sample preparation yielded positive real-time PCR results at 2 WPI (data not shown).

5. Discussion

The capacity of molecular methods for the early diagnosis of fasciolosis have recently been explored as an alternative to the coproELISA, with claims that *F. hepatica* "free worm" DNA can be detected in faecal samples as early as 1–2 WPI (Martinez-Perez et al., 2012; Martinez-Valladares and Rojo-Vazquez, 2016; Robles-Perez et al., 2013). Several molecular tools, including conventional and nested PCRs, as well as loop-mediated isothermal amplification (LAMP), have been shown to detect *F. hepatica* DNA in faecal samples prior to completion of the PPP (< 8 weeks) in experimental infections (Martinez-Perez et al., 2012; Martinez-Valladares and Rojo-Vazquez, 2016; Robles-Perez et al., 2013). It was proposed that successful amplification of DNA from faecal samples prior to the presence of *F. hepatica* eggs in faeces may be associated with cellular material from immature flukes, i.e. "free worm" DNA not contained within eggs (Martinez-Perez et al., 2012). Such "free worm" DNA potentially consists of cells sloughed from the integument of immature and adult flukes in response to the animal's immune response (Martinez-Perez et al., 2012). A real-time PCR diagnostic workflow has previously demonstrated good correlation with morphological FEC results, but it was not known if these results were influenced by the presence of "free worm" DNA (Calvani et al., 2017). The current study aimed to evaluate the contribution of *F. hepatica* "free worm" DNA to the real-time PCR results in experimentally infected sheep faecal samples while also comparing the initial point of detection of infection with *F. hepatica* by real-time PCR to a traditional sedimentation and a commercially available coproELISA (Happich and Boray, 1969; Mezo et al., 2004).

Several studies comparing LAMP to real-time PCR for the diagnosis of a variety of parasitic and viral diseases have demonstrated agreement between the two techniques (Lin et al., 2012; Sugiyama et al., 2005; Wang et al., 2013). To evaluate the capacity of the real-time PCR to detect *F. hepatica* DNA without the presence of eggs in faeces we compared two methods of sample preparation on faecal samples collected 2 WPI: (i) isolation of DNA from 150 mg raw faeces and (ii) isolation of DNA from an initial volume of 3 g of faeces subjected to a traditional sedimentation and pelleting (Calvani et al., 2017; Happich and Boray, 1969). In contrast to a previous study that employed 0.5 g raw faeces for amplification of *F. hepatica* DNA with LAMP and conventional PCR, neither of our sample preparation methods yielded positive results at 2 WPI using real-time PCR (Martinez-Valladares and Rojo-Vazquez, 2016). The poor sensitivity of both conventional PCR and LAMP when diagnosing naturally infected animals, has been suggested as possibly due to insufficient starting volume of faecal material and efficiency of current DNA extraction methods (Arifin et al., 2016). Our second approach addressed this issue by increasing the starting volume from the manufacturer-recommended 150 mg to 3 g prior to sedimentation and pelleting. Whilst this approach results in the concentration of eggs, increasing the sensitivity after completion of the PPP, it may inadvertently wash off any "free worm" DNA, resulting in false negative samples early on in the infection (Calvani et al., 2017). The earliest detection by real-time PCR using the sedimentation and pelleting approach for sample preparation occurred at 7 WPI and by 9 WPI all animals were considered PCR positive, suggesting that DNA detected by the current real-time PCR method is highly associated with the presence of *F. hepatica* eggs in faeces. A strong positive correlation between mEPG and qEPG (R^2 = 0.94) further suggests that our positive real-time PCR results are associated with faecal egg load, limiting its application to after completion of the PPP. Whether "free worm" *Fasciola* spp. DNA can be detected in faecal samples prior to the presence of eggs by other molecular approaches such as LAMP requires further scrutiny before they can be considered applicable in a diagnostic setting.

The traditional sedimentation results from the current study confirm the 8–12 week PPP for *F. hepatica* infection in Merino sheep and are in agreement with similar experimental infections of sheep, regardless of initial infective dose (Supplementary Table S1) (Brockwell et al., 2013; Flanagan et al., 2011; Martinez-Perez et al., 2012; Mezo et al., 2004; Valero et al., 2009). The majority of animals in this study (7/10) were positive by sedimentation as early as 8 WPI (mean FEC = 6 EPG) and all animals were positive by 9 WPI. Our results highlight the capacity of the sedimentation approach to identify infection in animals with low FECs (< 10 EPG) (Supplementary Table S1) (Calvani et al., 2017; Happich and Boray, 1969). The high sensitivity in samples with low FECs was achieved through duplicate sedimentations (2 × 3 g faecal starting volume per sample), increasing the diagnostic sensitivity from 33% to 66% (Calvani et al., 2017; Happich and Boray, 1969). High sensitivity is important in both early and low-level infections, particularly in cattle where the large volume of faeces and low numbers of eggs make diagnosis by this method difficult. However, despite the observable increase in sensitivity, the increase in processing time required for a dual sedimentation/FEC approach hinders its applicability as a medium to large-scale diagnostic tool (Supplementary Table S2).

Detection of coprological antigen provides an approach for the diagnosis of *Fasciola* spp. infection capable of giving positive results prior to the completion of the PPP, with increased sample throughput compared to the traditional sedimentation (Supplementary Table S2) (Gordon et al., 2012; Mezo et al., 2004). During development of a commercially-available MM3 antigen-based coproELISA for the diagnosis of *Fasciola* spp. infection, initial reports stated detection of coproantigen as early as 5 WPI in sheep, with 100% of animals positive at 10 WPI (Mezo et al., 2004). Since commercialisation of this coproELISA, several experimental infection studies have reported similar results, with a slight delay in detection of animals infected with *F. gigantica* (Supplementary Table S1) (Flanagan et al., 2011; Martinez-Perez et al., 2012; Valero et al., 2009). Our results are in agreement with other experimental infections of sheep, with the first detection occurring at 6 WPI in 30% of experimentally infected sheep, and 100% of animals positive by 9 WPI (Supplementary Table S1) (Brockwell et al., 2013; Valero et al., 2009). Issues with the coproELISA have arisen in the diagnosis of lighter infections, particularly in cattle, where the dilution of coproantigen in faeces reduces the OD below the manufacturer's recommended positive threshold (Brockwell et al., 2013; Calvani et al., 2017; Gordon et al., 2012; Kajugu et al., 2015; Novobilsky et al., 2012). It has been suggested that the large infective dose (≥200 metacercariae) used in many experimental infection studies may contribute to the disparity between the performance of the

coproELISA in experimental and natural infections (Gordon et al., 2012). An assessment of the capacity of the coproELISA against multiple life stages concluded that whilst it may be an appropriate tool for the monitoring of adult infections, it is not as reliable against immature stages of the parasite (George et al., 2017b). Subsequently, the performance of the coproELISA in the current study may not necessarily reflect its diagnostic sensitivity in a natural infection setting.

6. Conclusion

Both the real-time PCR and coproELISA are more convenient and allow greater sample throughput than the traditional sedimentation approach, particularly if dual sedimentation/FECs are required for increased sensitivity (Supplementary Table S2) (Calvani et al., 2017; George et al., 2017b; Gordon et al., 2012). An additional benefit of the DNA isolation includes the prolonged storage of samples for multiple uses, such as the potential for incorporation into existing diagnostic PCR panels such as those targeting sheep nematodes (Roeber et al., 2012; Roeber et al., 2017). A lack of detection of "free worm" DNA limits the capacity of the real-time PCR approach to diagnosis after completion of the PPP. Despite the fact that the real-time PCR failed to detect positive samples at 2 WPI, there was no significant difference between the diagnostic capacity of the three methods (sedimentation, coproELISA and real-time PCR) at 8 WPI in an experimental infection setting.

Conflict of interest statement

SG is a paid employee of ELANCO Animal Health, Australia. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2018.01.004.

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Chapter 4

A quick and simple benchtop vortex egg-disruption approach for the molecular diagnosis of *Fasciola hepatica* from ruminant faecal samples

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Nichola Calvani in the preparation and submission of the following manuscript:

> Calvani, N.E.D., Cheng, T., Green, C., Hughes, P., Kwan, E., Maher, E., Bush, R.D. and Šlapeta, J. (2018). A quick and simple benchtop vortex egg-disruption approach for the molecular diagnosis of *Fasciola hepatica* from ruminant faecal samples. Parasitology Research 117 (8): 2685-2688.

Contribution to the manuscript of all authors is as follows:

Task	**Contributing Co-authors**
Conceptualisation	NEDC, JŠ
Data collection and curation	NEDC, TC, CG, PH, EK, EM, JŠ
Formal analysis	NEDC, TC, CG, PH, EK, EM
Funding acquisition	RDB, JŠ
Investigation	NEDC, TC, CG, PH, EK, EM, JŠ
Methodology	NEDC, TC, CG, PH, EK, EM
Supervision, discussion and consultation	RDB, JŠ
Preparation of figures used in the manuscript	NEDC, JŠ
Writing of the original draft	NEDC
Critical review and editing of the manuscript	NEDC, RDB, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct. I have sighted email or other correspondences from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Kind regards,

Assoc. Prof. Russell Bush
14th January, 2020

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SHORT COMMUNICATION

A quick and simple benchtop vortex egg-disruption approach for the molecular diagnosis of *Fasciola hepatica* from ruminant faecal samples

Nichola Eliza Davies Calvani[1,2] · Tina Cheng[1] · Christine Green[1] · Patrick Hughes[1] · Emily Kwan[1] · Elizabeth Maher[1] · Russell David Bush[2] · Jan Šlapeta[1]

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Abstract
Commonly employed diagnostic methods for *Fasciola* spp., such as a traditional sedimentation and faecal egg count, or a commercially available coprological ELISA, have limitations in their sensitivity or ability to differentiate species. A reliable DNA isolation method coupled with real-time PCR addresses these issues by providing highly sensitive and quantitative molecular diagnosis from faecal samples. The current study evaluated a standard benchtop vortex for *F. hepatica* egg disruption in sheep and cattle faecal samples and determined the minimum faecal egg load required for a positive result from un-concentrated (raw) faecal samples. The minimum faecal egg load for a positive real-time PCR result from 150 mg raw faecal sample was 10 and 20 eggs per gram for sheep and cattle, respectively. No significant difference ($P = 0.4467$) between disruptions on a benchtop vortex for 5 or 10 min was observed when compared to 40 s of disruption at 6.0 m/s in a benchtop homogeniser.

Keywords DNA isolation · Fasciolosis · Molecular diagnostics · Real-time PCR · Vortex

Introduction

Fasciolosis is a production-limiting zoonotic disease of ruminants caused by infection with *Fasciola hepatica*, *Fasciola gigantica* and their hybrid forms (Torgerson and Macpherson 2011). Ante-mortem diagnosis is commonly performed by a traditional sedimentation and faecal egg count, or more recently via detection of coprological antigen with a commercially available ELISA that enables diagnosis during the pre-patent period (Happich and Boray 1969a; Mezo et al. 2004). Overlaps in egg morphology and excretion of the same antigen prevent species differentiation in areas of sympatry, an issue that molecular diagnosis has the capacity to resolve (Mezo et al. 2004; Valero et al. 2009).

The application of a molecular diagnostic approach requires the use of a high-speed benchtop homogeniser and the inclusion of a time-consuming egg-concentration step, limiting its use in high-throughput laboratories (Calvani et al. 2017; Demeler et al. 2013; Roeber et al. 2012; Roeber et al. 2017). The current study aims to evaluate the disruption potential of a standard benchtop vortex fitted with a 24 1.5–2-ml tube adaptor as a potential alternative to the high-speed benchtop homogeniser for the disruption of *F. hepatica* eggs within ruminant faecal samples. The minimum faecal egg load required for positive real-time PCR results will be determined when DNA is isolated from 150 mg raw faeces as opposed to using the egg-concentration technique (sedimentation of 3 or 6 g, sheep and cattle respectively) with the aim of reducing sample processing complexity.

Tina Cheng, Christine Green, Patrick Hughes, Emily Kwan and Elizabeth Maher contributed equally to this work.

✉ Jan Šlapeta
jan.slapeta@sydney.edu.au

[1] Laboratory of Veterinary Parasitology, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, NSW, Australia

[2] Mekong Livestock Research Group, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camperdown, NSW, Australia

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Materials and methods

Fasciola hepatica-spiked faecal samples

F. hepatica eggs collected from the gall bladder of an adult Merino sheep during routine post mortem examination were stored in phosphate buffered saline (PBS) solution (pH = 7.4) at 4 °C covered from light. Faecal samples were collected from Holstein-Friesian dairy cattle with no prior exposure to *F. hepatica* and spiked with a known number of clean *F. hepatica* eggs. Prior to spiking, faecal samples were confirmed to be free of *F. hepatica* eggs via a traditional sedimentation and faecal egg count (FEC) and a previously optimised real-time PCR for the diagnosis of *F. hepatica* in faecal samples (Calvani et al. 2017; Happich and Boray 1969a). For each of the spiked faecal samples, 150 mg *F. hepatica*-free faeces and 150 μl *F. hepatica* egg-PBS solution (equivalent to 200 eggs) was added to a 1.5-ml ceramic bead lysis tube along with 750 μl lysis buffer prior to DNA isolation.

DNA isolation

Three egg-disruption treatments were applied to the *F. hepatica*-spiked bovine faecal samples and compared to 40 s of homogenisation at 6.0 m/s on a FastPrep-24 benchtop homogeniser (MP Biomedicals, Australia). The three treatments consisted of disrupting the samples on a standard benchtop vortex (Chiltern MT19) fitted with a 24 1.5–2-ml tube adaptor (Cat No./ID: 13000-V1-24; Qiagen, Germany) for either (i) 40 s, (ii) 5 min or (iii) 10 min. Each disruption treatment was performed in duplicate and 5 μl DNA Extraction Control (BioLine, Australia) was added to the egg/faeces/lysis buffer mix to monitor for PCR inhibition. The remaining DNA isolation was performed according to the manufacturer's instructions (BioLine, Australia) and eluted in a final volume of 100 μl.

Real-time PCR

A TaqMan real-time PCR assay targeting *F. hepatica* ITS2 rDNA was utilised (oligonucleotides SSCPFaF [S0754]/SSCPFaR [S0755] and probe ProFh [S0770] FAM-BHQ1) and run in duplicate (Alasaad et al. 2011). All real-time PCR reactions were run on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia) using SsoAdvanced Universal Probes Supermix (BioRad, Australia) according to the manufacturer's instructions. The PCR mix included the probe and primers at a final concentration of 100 and 400 nM, respectively. Cycling conditions consisted of 3 min activation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Samples were run in duplicate and results were considered to be positive if both replicates displayed Ct values < 36. Extraction Control samples with Ct values < 31 were considered not inhibited. A tenfold serial dilution of DNA isolated from an adult *F. hepatica* fluke was used as the positive control and served to quantify egg estimates of EPG as described by Calvani et al. (2017). The EPG based on real-time PCR is henceforth referred to as qEPG.

Diagnostic application

To assess the diagnostic capacity of the optimised vortex disruption approach for the diagnosis of *F. hepatica* from 150 mg raw faeces, samples from naturally infected cattle ($n = 10$) and sheep ($n = 16$) were tested. All animals were located on a grazing property in Newcastle, New South Wales. Samples were collected by a licenced veterinarian during routine handling and sent to the Veterinary Parasitology Diagnostic Laboratory at The University of Sydney for testing. Prior to DNA isolation, samples were confirmed positive for *F. hepatica* by a traditional sedimentation and FEC (Happich and Boray 1969a). All morphological FECs based on microscopic examination are referred to as mEPG. For each animal, 150 mg raw faeces was added to a 1.5-ml ceramic bead lysis tube with 750 μl lysis buffer and 5 μl DNA Extraction Control (BioLine, Australia). Faecal samples were disrupted on a standard benchtop vortex for 10 min, after which the remainder of the DNA isolation was performed according to manufacturer's instructions, with DNA eluted in a final volume of 100 μl. DNA amplification was performed in duplicate as described in the previous section.

Statistical analysis and data availability

Data was analysed and visualised in GraphPad Prism version 7 (GraphPad Software, USA) using non-parametric tests (Kruskal-Wallis).

Results and discussion

Our results demonstrate that 5–10 min of vortexing will equally disrupt 200 *F. hepatica* eggs spiked in 150 mg bovine faecal samples for the purpose of DNA isolation and subsequent detection with real-time PCR when compared to disruption for 40 s in a high-speed benchtop homogeniser (Fig. 1) ($P = 0.4467$). Vortexing spiked faecal samples for 40 s only resulted in insufficient disruption of *F. hepatica* eggs for the purpose of DNA isolation (Fig. 1) ($P = 0.316$). While the use of a high-speed benchtop homogeniser enables rapid *Fasciola* spp. egg disruption, the need for non-standard and comparatively expensive laboratory equipment may be prohibitive for some diagnostic and research facilities (Calvani et al. 2017).

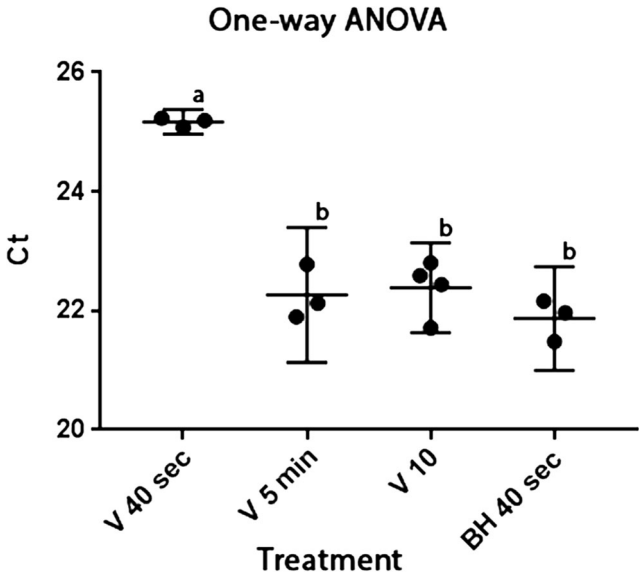

Fig. 1 Real-time PCR Ct values for disruption treatments applied to 150 mg bovine faecal samples spiked with 200 *F. hepatica* eggs. Three vortex (V) treatments (i. 40 s, ii. 5 min and iii. 10 min) were compared to 40 s of disruption in a benchtop homogeniser (BH) at 6.0 m/s. A significant difference ($P = 0.0316$) was observed between samples vortexed for 40 s and all other treatments, including the benchtop homogeniser (a). No significance ($P = 0.4467$) was observed between treatments ii. and iii. when compared to disruption in the benchtop homogeniser (b). Means and 95% confidence intervals are indicated for each treatment

Table 1 Morphological FECs and molecular estimates of *F. hepatica* EPG from sheep and cattle faecal samples

Species	Sample ID	mEPG	qEPG
Sheep	E10	1	–
	E11	1	–
	E15	1	–
	E16	2	–
	E8	4	–
	E14	5	–
	E1	13	1.8
	E5	17	2.6
	E2	34	0.9
	E6	50	6.4
	E9	60	12.1
	E12	70	22.9
	E4	72	15.9
	E3	82	25.7
	E13	99	17.5
	E7	128	14.4
Cattle	N12	1	–
	N7	2	–
	N1	3	–
	N14	6	–
	N6	10	–/–
	N8	20	16.1
	N2	70	16.5
	N3	95	–/67.0
	N4	130	21.4
	N5	220	18.7

mEPG, morphological faecal eggs count; qEPG, faecal egg count estimate based on quantitative real-time PCR results

Disruption using a standard benchtop vortex for 30 min in combination with glass beads has previously established the capacity of more accessible methods to rupture single *Fasciola* spp. eggs (Ai et al. 2010). The glass bead method was intended for research purposes only and hence was conducted in conjunction with a time-consuming washing-sieving procedure to free the eggs from faecal material, precluding its use in a high-throughput diagnostic laboratory setting (Ai et al. 2010; Suhardono et al. 2006). Our initial spiked sample results provided proof of principal that the more accessible standard benchtop vortex method for *F. hepatica* egg disruption enables substitution of a high-speed homogeniser, increasing the availability of this method of DNA isolation to a greater range of laboratories.

The above 10-min vortexing method was subsequently tested to determine if it also enables diagnosis of *F. hepatica* eggs directly from 150 mg of naturally infected cattle or sheep faecal samples. Faecal samples with a range of egg loads (1–128 and 1–220 EPG for sheep and cattle, respectively) were tested to determine the analytical sensitivity. The observed limit of detection (positive threshold) was 10 and 20 EPG for sheep and cattle, respectively (Table 1). When faecal egg load was above the positive threshold, *F. hepatica* eggs were successfully disrupted in 5/6 and 10/10 cattle and sheep faecal samples, respectively, thereby validating the egg-disruption capacity of a benchtop vortex on 150 mg raw faecal samples (Table 1). In routine diagnostic workflows, DNA isolation directly from raw samples is preferable, despite a slight decrease in analytical sensitivity. The positive threshold observed in the current study is considered sufficient for diagnosis because (i) 20 EPG is representative of a single fluke in the liver of sheep and (ii) a single *F. hepatica* fluke is capable of producing up to 25,000 eggs per day (Happich and Boray 1969b).

We have previously demonstrated a method of egg concentration capable of the highly sensitive (≤ 1 EPG) molecular diagnosis of *F. hepatica* applicable in circumstances where a very high analytical sensitivity is required, such as during epidemiological investigations or drug efficacy trials (Calvani et al. 2017). As predicted, when used on a 10 EPG bovine faecal sample (N6, Table 1), the concentration approach resulted in the successful detection of *F. hepatica* DNA (30.6 qEPG), yet failed to amplify *F. hepatica* DNA from the same sample without application of the concentration step (Table 1). In one instance, a bovine faecal sample with a high egg load

(95 EPG) failed to amplify without application of the concentration step (N3, Table 1). Re-isolation of DNA from this sample using both the raw 150-mg method and the egg-concentration approach resulted in successful amplification (67 and 553 qEPG, respectively), indicating that isolation of DNA from duplicate samples may occasionally be necessary (Calvani et al. 2017).

Traditional parasitological techniques for the identification of nematode and trematode species and genera in ruminant field samples typically involve the combination of three parasitological techniques—sedimentation, faecal floatation and larval culture. Larval culture requires 7–10 days for the development of L3 larvae, and morphological identification of both eggs and larvae has the potential for inaccuracies. In response to the need for a more rapid and definitive method of diagnosis, a faecal panel was developed for the detection of nematodes in Australia (Roeber et al. 2011). Despite its economic importance, *F. hepatica* was not included, potentially due to the initial flotation egg-concentration step which inherently excludes *F. hepatica*. Regardless, the streamlined DNA isolation protocol presented in the current paper enables incorporation of *F. hepatica* into existing multi-species faecal panels allowing differential diagnosis of a wider range of ruminant nematodes and trematodes in a single step.

We have demonstrated that use of a standard benchtop vortex results in consistent *F. hepatica* egg disruption for DNA isolation and amplification from faecal samples of sheep and cattle, employing more widely available laboratory equipment, while isolation of DNA from 150 mg raw faeces dramatically reduces sample processing time.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 5

Which species is in the faeces at a time of global livestock movements: SNP genotyping assays for the differentiation of *Fasciola* spp.

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Nichola Calvani in the preparation and submission of the following manuscript:

> Calvani, N.E.D., Ichikawa-Seki, M., Bush, R.D., Khounsy, S. and Šlapeta, J. Which species is in the faeces at a time of global livestock movements: SNP genotyping for the differentiation of *Fasciola* spp.. International Journal for Parasitology 50 (2): 91-101.

Contribution to the manuscript of all authors is as follows:

Task	**Contributing Co-authors**
Conceptualisation	NEDC, RB, JŠ
Data collection and curation	NEDC, MI
Formal analysis	NEDC, JŠ
Funding acquisition	RDB, JŠ
Investigation	NEDC, MI, JŠ
Methodology	NEDC, JŠ
Supervision, discussion and consultation	RDB, MI, SK, JŠ
Preparation of figures used in the manuscript	NEDC, JŠ
Writing of the original draft	NEDC
Critical review and editing of the manuscript	NEDC, MI, RDB, SK JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct. I have sighted email or other correspondences from all co-authors confirming their certifying authorship and permission for manuscript inclusion in this thesis.

Kind regards,

Assoc. Prof. Russell Bush
15th May, 2020

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Which species is in the faeces at a time of global livestock movements: single nucleotide polymorphism genotyping assays for the differentiation of *Fasciola* spp. ☆

Nichola Eliza Davies Calvani [a,b], Madoka Ichikawa-Seki [c], Russell David Bush [b], Syseng Khounsy [d], Jan Šlapeta [a,*]

[a] Laboratory of Veterinary Parasitology, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Australia
[b] Mekong Livestock Research Group, Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Australia
[c] Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, Japan
[d] Department of Livestock and Fisheries, Lao Democratic People's Republic

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ABSTRACT

Fasciolosis, caused by *Fasciola hepatica* and *Fasciola gigantica,* is a globally distributed zoonotic disease of livestock. While *F. hepatica* and *F. gigantica* have temperate and tropical distributions, respectively, parasite sympatry occurs in parts of Asia and Africa. A growing protein demand has the potential to facilitate the translocation of parasites from endemic to non-endemic areas, via associated international livestock movements. Such is the case in Southeast Asia, where livestock trade from *F. hepatica*-endemic countries into China and Vietnam may account for detection of *F. hepatica* hybrid/introgressed forms. Of particular importance is Lao People's Democratic Republic, which acts as a major livestock thoroughfare for the region. Our ability to understand the impacts of livestock-associated *Fasciola* spp. movements on local animal and human health is hindered by a lack of ante-mortem diagnostic tools allowing species differentiation. Molecular tools have been developed for *Fasciola* spp. differentiation, however those rely on access to pure DNA from adult specimens, limiting their application to post-mortem use. Our aim was to detect and differentiate *F. hepatica* from the endemic *F. gigantica* in local smallholder cattle in a region of Southeast Asia with frequent livestock trafficking. To do this we designed and validated ante-mortem molecular assays for *Fasciola* spp. differentiation targeting single-nucleotide polymorphisms (SNPs) within ITS1 and lsrRNA. We then deployed these SNP genotyping assays to diagnose *Fasciola* spp. infection in 153 local cattle from 27 villages in Northern Laos. We demonstrate the presence of *F. hepatica* DNA, confirmed by qualitative Sanger and quantitative Illumina amplicon sequencing of ITS1 and lsrRNA, and highlight the shortfalls of Sanger sequencing for *Fasciola* spp. identification due to the preferential amplification of *F. gigantica* nucleotides in mixed DNA samples. The outlined protocol enables rapid surveillance of faecal samples for the presence of *Fasciola* species eggs, their co-infection and/or infection with *F. hepatica*/*F. gigantica* hybrids.

1. Introduction

Fasciolosis is a zoonotic disease of livestock with over 91 million people considered at risk of infection globally and economic impacts estimated to exceed US $3 billion/year (Keiser and Utzinger, 2005; Torgerson and Macpherson, 2011). Fasciolosis is caused by infection with either *Fasciola hepatica* or *Fasciola gigantica* in temperate and tropical regions, respectively (Mas-Coma et al., 2009). Despite the significant human and animal health impacts, it remains a neglected disease, especially in developing countries (Cwiklinski et al., 2016; Stothard et al., 2018). In livestock, infection results in decreased production outcomes such as reduced weight gain and body condition scores, poor carcass quality, decreases in reproductive performance and, in acute cases, sudden death (Dalton, 1999; Mas-Coma et al., 2014). Clinical signs in humans are associated with damage and fibrosis of the liver, resulting in acute to chronic abdominal pain, fever, loss of appetite, anaemia and diarrhoea (Arjona et al., 1995; Chen et al., 2013; da

☆ Note: Nucleotide sequence data reported in this paper are available in GenBank under accession numbers MN821532-35 and MN821559-64.

* Corresponding author.
E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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Costa et al., 2019). In susceptible hosts, *F. hepatica* undergoes an 8–12 week pre-patent period (PPP), a significant portion of which involves the destructive migration through the liver (Dalton, 1999). Upon maturation in the bile ducts and gall bladder, the hermaphroditic flukes shed up to 24,000 eggs per day for over a year (Happich and Boray, 1969b). Due to its larger size, *F. gigantica* may be considered more pathogenic at the individual level compared with *F. hepatica*, causing significant damage to the liver of infected hosts during a longer (12–16 week) PPP (Dalton, 1999; Valero et al., 2016). On the other hand, *F. hepatica* has shown greater infectivity by establishing larger burdens in definitive hosts, is reported to have developed widespread anthelmintic resistance and it is believed to have a greater zoonotic potential (Taira et al., 1997; Dalton, 1999; Valero et al., 2016; Toldedo and Freid, 2019).

Translocation of *Fasciola* spp. from endemic to non-endemic areas has long been associated with global livestock movements (Mas-Coma et al., 2009). Recently, a growing middle class in countries such as China and Vietnam has seen protein demands far outweigh local production, resulting in the importation of 150,000 animals into China per month in 2019 (USDA, 2019). Over 70% of these animals were imported from *F. hepatica*-endemic countries including Brazil, Argentina and Australia (Dalton, 1999; Mas-Coma et al., 2009; USDA, 2019). The ability of *F. hepatica* to colonise new environments is well known, thus the transportation of infected animals has implications for the distribution of *F. hepatica* throughout the region (Gasnier et al., 2000; Mas-Coma et al., 2005). The importation of *F. hepatica* is of particular relevance in the People's Democratic Republic of Laos (Lao PDR), due to its role as a major livestock thoroughfare from *F. hepatica*-endemic countries into both China and Vietnam (Smith et al., 2015). Documentation of livestock movements in Lao PDR is limited, however the unregulated movement of cattle and buffalo from Northern Laos into China was estimated to have exceeded 58,000 head in 2014, with that number likely to have increased since (Smith et al., 2015).

The impact of the trade of livestock in Southeast Asia on *Fasciola* spp. infection in local animals remains unknown. Few studies have conducted molecular identification of *Fasciola* spp. in the region, although there is evidence to suggest the recent introduction of *F. hepatica* DNA via the detection of hybrid/introgressed forms into both Thailand and Vietnam (Bui et al., 2016; Le et al., 2008; Wannasan et al., 2014; Salzer and Schmiedel, 2015). Prevalence data is often restricted to abattoir surveillance, making it difficult to draw epidemiological conclusions beyond the province level due to a lack of animal traceability (Sothoeun et al., 2006; Rast et al., 2013; Smith et al., 2015). The absence of more detailed field studies is in part due to a lack of tools enabling wide-scale ante-mortem species differentiation (Ai et al., 2011; Cwiklinski et al., 2016). Traditionally, infections are diagnosed using time-consuming microscopy-based methods, which cannot reliably differentiate parasite species due to overlapping egg morphology (Valero et al., 2009a). Immunological techniques such as commercially-available ELISAs enable detection of infection prior to the PPP as well as increased sample throughput, but do not allow species differentiation (Valero et al., 2009b). Species differentiation using molecular tools is becoming increasingly common worldwide, however relies on access to pure DNA sourced from adult parasites in the liver and is therefore limited to post-mortem surveillance (Agatsuma et al., 2000; Ai et al., 2011; Nguyen et al., 2012).

Our ultimate aim was to develop a tool enabling wide-scale ante-mortem *Fasciola* spp. surveillance and species differentiation beyond the abattoir in areas of frequent livestock thoroughfare. This was first achieved by the development and optimisation of two TaqMan probe-based real-time PCR (qPCR) assays targeting species-specific single nucleotide polymorphisms (SNPs) with ITS1 and lsrRNA for the qualitative differentiation of *Fasciola* spp. in faecal samples. We then aimed to determine the potential impacts of international livestock movements on local cattle by using these qPCR SNP genotyping assays to screen field samples collected from cattle in Northern Laos. Our final goal was to then discern the quantitative contributions of each *Fasciola* spp. within a given sample via the development of Next Generation Sequencing (NGS) assays and compare species differentiation using these ITS1 and lsrRNA Illumina amplicons to traditional Sanger sequencing.

2. Material and methods

2.1. Fasciola spp. samples

2.1.1. DNA from adults

Morphologically identified adult *F. hepatica* from Australia and *F. gigantica* from Laos obtained in 2016 and 2017, respectively, were sourced from the parasite collection at the Sydney School of Veterinary Science, University of Sydney, Australia (Calvani et al., 2017). Specimens of *F. hepatica*/*F. gigantica* hybrid adults (*Fasciola* hybrids) were from the experimental strains maintained at Iwate University, Japan (Itagaki et al., 2011). Total genomic DNA was isolated from 25 mg of each species of adult fluke using the Isolate II Genomic DNA kit (BioLine, Australia) according to the manufacturer's instructions and eluted in 100 µl of elution buffer (10 mM TrisCl buffer, pH = 8.5). The initial DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Australia) before being diluted 1:100 with ddH_2O and stored at −20 °C (LabArchives: Supplementary Data S1).

2.1.2. DNA from eggs and faecal samples

DNA from *F. hepatica* (n = 13) and *F. gigantica* (n = 8) positive faecal samples was sourced from a previous study (Supplementary Data S1) (Calvani et al., 2017, 2018). DNA from specimens of *Fasciola* hybrid eggs in faecal samples from Wistar rats from Iwate University, Japan was isolated from half of a rat faecal pellet using the Isolate Faecal DNA kit (Bioline, Australia) following the manufacturer's recommendations with the exception of the vortexing step which was conducted on a benchtop homogeniser as previously described (Calvani et al., 2018). DNA was isolated into 100 µl of elution buffer and stored at −20 °C.

Eggs from *F. hepatica* and *F. gigantica* were manually removed from the adults of each species and 10, 50 and 90 *F. hepatica* eggs were mixed in lysis buffer with 90, 50 and 10 *F. gigantica* eggs (1:9, 1:1 and 9:1 ratios of *F. hepatica* to *F. gigantica* eggs) before mechanical lysis on a benchtop homogeniser and DNA isolation using the Isolate Faecal DNA kit (Bioline, Australia) as described above.

2.1.3. Molecular confirmation of Fasciola spp. adults via existing conventional genotyping PCRs

Three existing conventional PCR assays were used for species confirmation of the adult specimens (Marcilla et al., 2002; Itagaki et al., 2005; Shoriki et al., 2016). A 639 bp region of nuclear ribosomal internal transcribed spacer 1 (ITS1) and a 577 bp fragment of 28S large subunit (LSU) ribosomal DNA (lsrRNA) within the rDNA unit, respectively, were amplified as previously described (ITS1: S0762/S0763; lsrRNA: S0756/S0757, Table 1) (Calvani et al., 2017).

DNA fragments of a single copy novel nuclear marker of phosphoenolpyruvate carboxykinase (PEPCK) were amplified using a single step multiplex PCR targeting 241 bp and 509 bp fragments of *F. hepatica* and *F. gigantica*, respectively (Fh-pepck-F/Fg-pepck-F/Fcmn-pepck-R, Table 1). The multiplex reactions for PEPCK were made up to 30 µl, including 10 µM of each primer, 15 µl of MyTaq Red Mix (BioLine, Australia) and 2 µl of template DNA. Reactions were run with an initial denaturation step at 95 °C for 90 s, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C

Table 1
Summary of primers amplifying *Fasciola hepatica* and *Fasciola gigantica* genes.

Loci	Assay	ID	Primer ID: Sequences	Reference
ITS1	Conventional & real-time PCRs	S0762	Forward: 5′-TTG CGC TGA TTA CGT CCC TG-3′	Itagaki et al., 2005
		S0763	Reverse: 5′-TTG GCT GCG CTC TTC ATC GAC-3′	
		S0808	Forward: 5′-TTA AAG AGG AGA TTT GGG C-3′	This study
		S0809	Forward: 5′-AGT GCT AGG CTT AAA GAG-3′	
		S0811	Reverse: 5′-CAA AGA CCA GGT TAT CAG-3′	
	SNP genotyping assay	Applied Biosystems ID: ANDJYUR	Forward: 5′-GCC AGG AGA ACG GGT TGT A-3′	This study
			Reverse: 5′-CGT AGC CCA AAT CTC CTC TTT AAG C-3′	
			Pro_Fh (FAM): 5′-CAC TAC CAA TCA TGG CAG-3′	
			Pro_Fg (VIC): 5′-TAG CAC TAC CAA TCG TGG CAG-3′	
	NGS	S0921	Forward: 5′-*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG*-TTA AAG AGG AGA TTT GGG C-3′	This study
		S0922	Forward: 5′-*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG*-AGT GCT AGG CTT AAA GAG-3′	
		S0923	Reverse: 5′-*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*-CA AAG ACC AGG TTA TCA G-3′	
ITS2	Real-time PCR	S0754	Forward: 5′-TTG GTA CTC AGT TGT CAG TGT G-3′	Alasaad et al., 2011
		S0755	Reverse: 5′-AGC ATC AGA CAC ATG ACC AAG-3′	
		S0770	Pro_Fh (FAM): 5′-ACC AGG CAC GTT CCG TCA CTG TCA CTT T-3′	
		S0771	Pro_Fg (HEX): 5′-ACC AGG CAC GTT CCG TTA CTG TTA CTT TGT C-3′	
lsrRNA	Conventional & real-time PCRs	S0756	Forward: 5′-ACG TGA TTA CCC GCT GAA CT-3′	Marcilla et al., 2002
		S0757	Reverse: 5′-CTG AGA AAG TGC ACT GAC AAG-3′	
		S0806	Reverse: 5′-CAA GCA GGT CCG TCA GTA-3′	This study
		S0807	Reverse: 5′-CAC CTG ATG AGT GAT TCG-3′	
		S0926	Forward: 5′-GAG TAA ACA GTG CGT GAA A-3′	
	SNP genotyping assay	Applied Biosystems ID: ANCE49U	Forward: 5′-TGC TCG GGT CTG CTT AGC-3′	This study
			Reverse: 5′-CAC AAC ACC TGA TGA GTG ATT CG-3′	
			Pro_Fh (VIC): 5′-TGC AGG TCT CCG CTT C-3′	
			Pro_Fg (FAM): 5′-CAG GTC CCC GCT TC-3′	
	NGS	S0925	Forward: 5′-*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAG*-TAA ACA GTG CGT GAA A-3′	This study
		S0920	Reverse: 5′-*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*-CA AGC AGG TCC GTC AGT A-3′	
PEPCK	Conventional PCR	Fh-pepck-F	Fh Forward: 5′-GAT TGC ACC GTT AGG TTA GC-3′	Shoriki et al. (2015)
		Fg-pepck-F	Fg Forward: 5′-AAA GTT TCT ATC CCG AAC GAA G-3′	
		Fcmn-pepck-R	Reverse: 5′-CGA AAA TTA TGG CAT CAA TGG G-3′	

Illumina overhang sequences are shown in italics.
ITS1, Internal Transcribed Spacer 1 ribosomal RNA gene; ITS2, Internal Transcribed Spacer 2 ribosomal RNA gene; lsrRNA, Large Sub Unit ribosomal RNA gene; PEPCK, Phosphoenolpyruvate caroxykinase; SNP, single nucleotide polymorphism; NGS, Next Generation Sequencing.

for 60 s, and a final extension step of 72 °C for 10 min, with the resultant products separated by electrophoresis and visualised on a 1% agarose gel.

All PCRs that yielded unambiguous single bands of the expected size were directly and bidirectionally sequenced using amplification primers at Macrogen Ltd. (Seoul, Korea), and assembled and compared with reference sequences for *F. hepatica* and *F. gigantica* (ITS1, AB514849 and KF543340; LSU, AY222244.1 and AJ439739) in CLC Main Workbench 6.9.1 (Qiagen, CLC Bio).

2.2. Identification of SNPs differentiating F. hepatica and F. gigantica

All available sequences of *Fasciola* spp. matching ITS1 and lsrRNA were retrieved on September 22, 2019 from GenBank by searching for *Fasciola* AND (ITS1 OR internal transcribed spacer 1) and *Fasciola* AND ((LSU OR large subunit) NOT (*Onchocerca* OR *Dictyota*)), respectively, and aligned using CLC Main Workbench 6.9.1. (Qiagen, CLC Bio, Australia). The search yielded 1146 and 1034 sequences, of which 867 and 239 for ITS1 and lsrRNA, respectively, were used for SNP identification. Prior to SNP identification, all non-*Fasciola* spp. sequences were removed, together with any sequences not spanning the area of interest (a total of 279 and 795 were removed for ITS1 and lsrRNA, respectively). Finally, any ambiguous or poorly aligned sequences were manually checked before SNPs consistently differentiating both species were identified via visual inspection of the final alignments.

2.3. TaqMan probe-based SNP genotyping assays for the differentiation of Fasciola spp.

Using the above alignments and within the boundaries set by our genotyping conventional PCR amplicons, we designed and ordered two custom TaqMan probe-based SNP genotyping assays targeting residue 383 and 513 of the ITS1 and lsrRNA, respectively (Thermo-Fisher Scientific, Australia) (unique assay IDs ITS1: ANDJYUR; lsrRNA: ANCE49U, Table 1).

Real-time PCRs were run according to the manufacturer's recommendations (Thermo-Fisher Scientific, Australia) using *Sso*Advanced Universal Probes Supermix (BioRad, Australia) on the CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Maestro 1.0 software (BioRad, Australia). The volumes of the real-time PCRs were made up to 10 µl, including 1 µl of template DNA, and primers and probes were used at a concentration of 900 nM and 200 nM, respectively. PCRs were initiated at 95 °C for 3 min, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. Each reaction contained DNA from *F. hepatica, F. gigantica* and hybrid adult flukes as positive controls, while ddH_2O acted as a negative control in each run.

The efficiency and limit of detection of both the ITS1 and lsrRNA SNP genotyping assays was determined by the generation of standard curves using serial 10-fold dilutions of DNA from the *F. hepatica*, *F. gigantica* and *Fasciola* hybrid adults from 1.75×10^{1} to 1.75×10^{-6} ng/µl using CFX Mestro 1.0 Software (BioRad Australia). Allelic specificity was tested by comparing the discrimination of *F. hepatica*- and *F. gigantica*-positive faecal samples against the positive controls (DNA from the morphologically and molecularly identified adult flukes). Assay specificity was also confirmed against *Fasciola*-negative faecal samples from Merino sheep ($n = 6$) known to be naturally infected with a range of other parasites (*Haemonchus*, *Trichostrongylus*, *Teladorsagia*, *Oesophagostomum, Cooperia*) (Supplementary Data S1).

2.4. Conventional PCRs for use on Fasciola-positive faecal samples

To specifically amplify DNA from faecal samples, reverse conventional PCR primers amplifying 617 and 570 bp fragments of

ITS1 and lsrRNA, respectively, were designed by alignment of *F. hepatica* and *F. gigantica* sequences from the GenBank database, using CLC Main Workbench 6.9.1 (Qiagen, CLC Bio). These new reverse primers were used in conjunction with existing forward primers, with bands visualised on 1.5% agarose gel (ITS1: S0762/S0811; lsrRNA: S0756/S0807, Table 1).

Conventional PCRs were performed using MyTaq Red Mix (BioLine, Australia) in a total volume of 30 μl, including a 2 μl template. Primers were added at a concentration of 250 and 125 nM each for lsrRNA and ITS1, respectively. The lsrRNA reactions were run using the following conditions: 95 °C for 15 s, 61 °C for 15 s and 72 °C for 20 s for 35 cycles. All reactions were initiated at 95 °C for 2 min and concluded at 72 °C for 7 min. The ITS1 reactions were the same, with the exception of the annealing temperature (59 °C). PCRs were amplified in the T100TM Thermal Cycler (BioRad, Australia). All reactions included ddH_2O as negative controls. Products were visualised on 1.5% agarose gel after electrophoresis.

2.5. Illumina PCR amplicon Next Generation Sequencing (NGS)

Amplification primers with Illumina adaptors were designed to amplify ~200 bp regions spanning specific SNP residues (ITS1: S0922/S0923 and S0921/S0923; lsrRNA: S0925/S0920, Table 1). The NGS first stage PCRs were performed in house according to the Illumina MiSeq 16S Library Preparation (Illumina, Australia) recommendations, with starting DNA concentrations determined based on quantification by qPCR (ITS1: S0809/S0811 and S0808/S0811; lsrRNA: S0926/S0806, Table 1; LabArchives: Supplementary Data S2). Cycling conditions were as recommended by Illumina, namely 95 °C for 3 min, followed by either 25 or 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s depending on the DNA starting concentration, and a final extension of 72 °C for 5 min (Supplementary Data S2). Pooled PCR amplicons were submitted for barcoding, library preparation using Illumina Nextera XT V2 and sequencing on an Illumina iSeq 100 System with 2 × 150 bp was run at the Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia. For SNP analysis, paired reads were merged using PEAR 0.9.6 (Zhang et al., 2014). Paired sequence reads were aligned to reference sequences using Burrows-Wheeler Aligner (BWA 0.7.17) (Li and Durbin, 2009). Variants were identified using Samtools 1.9 (Li et al., 2009), SNPs were visualised and the nucleotide frequencies of both the *F. hepatica* and *F. gigantica* SNPs were exported using IGV 2.5.3 (http://broadinstitute.org/igv). As an alternative approach to SNP analysis, we utilised the DADA2 pipeline (https://benjjneb.github.io/dada2/; package version 1.12.1) to infer the exact amplicon sequence variants (ASVs) from the amplicon data (Callahan et al., 2016). All sequence data was filtered and denoised per forward and reverse read, trimmed to 130 nucleotides (nt) based on quality scores and merged, before chimeras were removed. In summary, >94% of input reads were kept and only 0.2% of the merged paired reads were removed as chimeras. Tabulated ASVs were then compared with reference genotypes of ITS1 and lsrRNA from *F. hepatica* and *F. gigantica*, where perfect and imperfect matches were identified based on pairwise comparison. For ITS1 ASVs, only those with 10 or more sequence reads were kept, while for lsrRNA all were kept. All NGS data analysis was performed on the Artemis HPC (Sydney Informatics Hub, The University of Sydney).

2.6. Fasciola spp. differentiation from cattle faecal samples collected in an area of frequent livestock thoroughfare

A total of 153 faecal samples were collected from local cattle in Northern Laos between July 2018 and February 2019. During the 2018 wet season, 15 cattle were sampled per village from five villages (total $n = 75$) (LabArchives: Supplementary Data S3). Samples were sedimented in Laos and the resultant sediment was stored in ≥96% (v/v) ethanol before transport to the Laboratory of Veterinary Parasitology, The University of Sydney, Australia for counting and molecular diagnosis (Happich and Boray, 1969a; Calvani et al., 2017). An additional 78 faecal samples were opportunistically collected during Foot and Mouth Disease surveillance from 22 villages in Northern Laos during the 2019 dry season ($n = 78$) (Supplementary Data S3). Samples were sedimented in Laos and two drops of sediment were briefly examined for eggs before the remaining sediment was stored in ≥96% (v/v) ethanol for transport to the Laboratory of Veterinary Parasitology, The University of Sydney, Australia for molecular diagnosis (Happich and Boray, 1969a; Calvani et al., 2017).

DNA was isolated from the entire faecal sediment using the Isolate Fecal DNA kit (Bioline, Australia) as previously described (Calvani et al., 2017). To monitor DNA isolation efficiency and the absence of PCR inhibition, 5 μl of DNA Extraction Control 670 (Bioline, Australia) was included in all samples, which were assayed according to the manufacturer's instructions on a CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Maestro 1.0 software (BioRad, Australia) using SensiFAST Probe No-ROX Mix (BioLine, Australia) with expected cycle threshold (C_t) values <31. Each DNA isolation batch included a blank sample (ddH2O) to detect any potential contamination during the extraction process (extraction negative control). All DNA was stored at −20 °C prior to molecular analysis.

The samples were tested for the presence of *Fasciola* spp. DNA and species differentiation was conducted using the two SNP genotyping assays as described in Section 2.3 (ITS1: ANDJYUR; lsrRNA: ANCE49U, Table 1). The faecal samples were additionally tested using a TaqMan real-time PCR targeting a 140 bp region of ITS2 with *F. hepatica* and *F. gigantica* polymorphic regions as previously described (S0754/S0755; ProFh S0770; ProFg S0771, Table 1) (Alasaad et al., 2011).

2.7. Data accessibility

Raw fastq sequence data was deposited at SRA NCBI BioProject: PRJNA564418. The nucleotide sequence data generated in this study was deposited in GenBank: accession numbers MN821532-35 and MN821559-64. Associated supplementary and additional data is available at LabArchives (https://doi.org/10.25833/rmar-x249).

3. Results

3.1. SNPs enable Fasciola spp. differentiation from adult DNA but do not clearly identify Fasciola hybrids

To screen a large number of animals for the presence of *Fasciola* spp., we needed to validate an approach that would be both sensitive and species-specific. To do this, we first interrogated publicly available sequences to identify markers likely to differentiate the two *Fasciola* spp. Two markers appeared as the most frequently used; ITS1 with 867 sequences with six consistent SNPs and partial lsrRNA with 239 sequences with two consistent SNPs (LabArchives: Supplementary Data S4).

We then focused on three SNPs distinguishing *F. hepatica* and *F. gigantica* at the ITS1 (residues 383, 461 and 481, relative to the ITS1 amplicon; primers: S0762/S0763, Table 1) and one SNP at the partial lsrRNA (residue 513, relative to the lsrRNA amplicon; primers S0756/S0757, Table 1). Species identification via conventional PCR amplification and Sanger sequencing of ITS1 and partial lsrRNA from *F. hepatica* and *F. gigantica* adult flukes confirmed unambiguous nucleotides at those residues (accession numbers MF678649, MF678650, MF678653 and MF678654) (Fig. 1A, B).

Amplification of DNA from a laboratory maintained *Fasciola* hybrid adult demonstrated ambiguous residues at all three of the ITS1 SNPs and at the one partial lsrRNA SNP (Fig. 1A, B). The double-peaked residues for the *Fasciola* hybrid appeared when the PCR was run for 28 cycles only and were dominated by *F. gigantica* nucleotides. The *F. hepatica* nucleotides were not visible in the *Fasciola* hybrid DNA sequences when the PCR was run for 35 cycles (Fig. 1A, B).

All three adult specimens were confirmed to be *F. hepatica, F. gigantica* or the *Fasciola* hybrid by detection of a single copy novel nuclear marker (PEPCK) (Fig. 2). Further confirmation was conducted via Sanger sequencing, where these amplicons showed 100% similarity to reference sequences (accession numbers LC061148 and LC061154) (Shoriki et al., 2016).

3.2. TaqMan probe-based SNP genotyping assays differentiate *F. hepatica* and *F. gigantica* DNA from Fasciola hybrid DNA from adults and faecal samples

Differentiation of *Fasciola* hybrid DNA from that of *F. hepatica* and *F. gigantica* was achieved via the development of SNP genotyping assays targeting residue 383 within the ITS1 and residue 513 within the partial lsrRNA. After manual allocation against the positive controls, 13/13 and 6/8 *F. hepatica* and *F. gigantica* known-positive faecal samples and five negative faecal samples formed species-specific clusters (Supplementary Data S1, Fig. 3A). The *F. hepatica* and *F. gigantica* faecal samples ranged in faecal egg counts from 0–1043 eggs per gram of faeces (EPG) and 0.5–14 EPG (Supplementary Data S1). The two samples that failed to amplify (NC17_001 and NC17_003) were *F. gigantica* faecal samples with only 0.5 EPG each. The 0 EPG (sedimentation-negative) *F. hepatica* faecal sample was from an experimentally-infected sheep 7 weeks p.i. and was a known PCR-positive sample from a previous study (Calvani et al., 2018). The *Fasciola* hybrid faecal sample from a laboratory maintained Wistar rat fell within the appropriate cluster as defined by the *Fasciola* hybrid adult DNA (Fig. 3A).

In both assays the angle of separation between *F. hepatica* and the *Fasciola* hybrid clusters was well-defined, however the angle of separation between *F. gigantica* and the *Fasciola* hybrid clusters was narrow (Fig. 3A). Regardless, the correct species were able to be differentiated across both assays, and lsrRNA showed the greatest degree of separation between the clusters for all species (Fig. 3A). No amplification was observed in any of the negative control samples. Both assays were highly efficient in detecting *Fasciola* spp. DNA between concentrations of 1.75×10^{1} to 1.75×10^{-5} ng/μl, with efficiencies between 90 and 110% for all fluorophores (Fig. 3B).

To confirm the allelic discrimination plot clustering, we used new conventional PCR assays for the *Fasciola*-specific amplification of DNA from faecal samples for ITS1 and partial lsrRNA for analysis using Sanger sequencing. Primers previously used in conjunction with conventional PCR to genotype adults from pure DNA (ITS1: S0762/S0763; lsrRNA: S0756/S0757, Table 1) produced non-

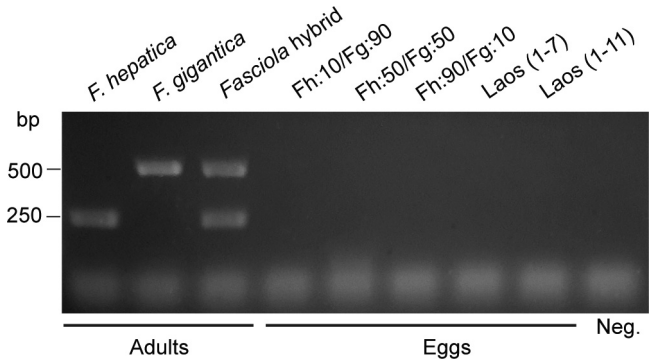

Fig. 2. *Fasciola* spp. identification via detection of a single copy novel nuclear marker (PEPCK). Lanes 1 to 3: band patterns of *Fasciola hepatica, Fasciola gigantica* and *Fasciola* hybrid adults; lanes 4 to 6: lack of detection of three mixed *F. hepatica* (Fh)/*F. gigantica* (Fg) egg samples at ratios of 1:9 (Fh:10/Fg:90), 1:1 (Fh:50/Fg:50) and 9:1 (Fh:90/Fg:10); lanes 7 to 8: lack of detection of *Fasciola* spp. DNA from two faecal samples from the Laos survey (Laos 1–7; Laos 1–11). Lane 9: no template control (Neg). Sample names are consistent with those in LabArchives Supplementary Data S1.

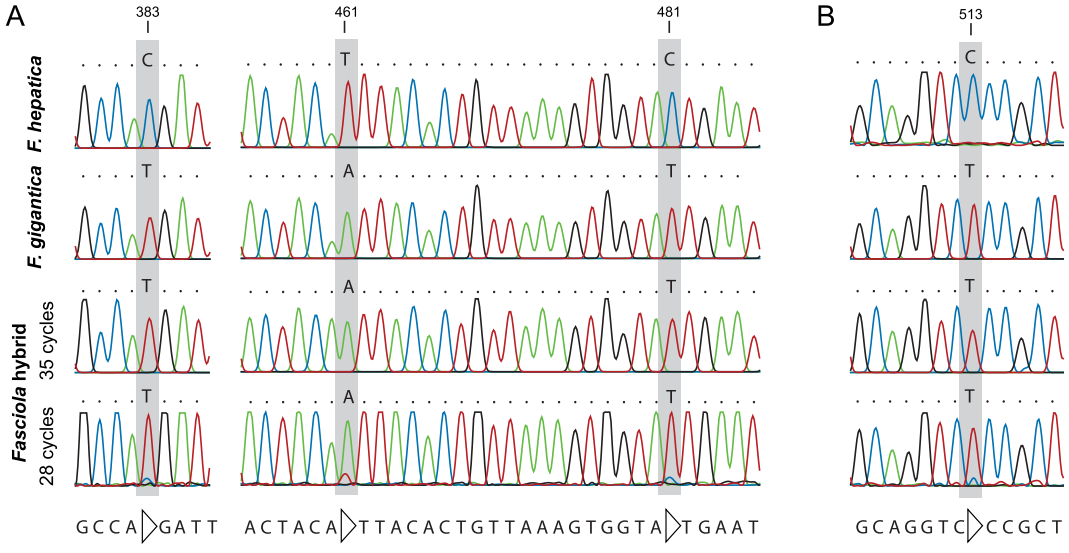

Fig. 1. Distinct nucleotide residues of *Fasciola hepatica*, *Fasciola gigantica* and a *Fasciola* hybrid in ITS1 and lsrRNA. DNA from morphologically identified adult specimens was PCR amplified and Sanger sequenced. The chromatographs show distinct single nucleotide polymorphisms (SNPs) between *F. hepatica* and *F. gigantica*. Residues 383, 461 and 481 relative to the ITS1 amplicon (A), and residue 513 relative to the lsrRNA amplicon (B) are shaded. Amplicons of a *Fasciola* hybrid amplified using 35 PCR cycles demonstrated matching residues with *F. hepatica*, but lowering the number of PCR cycles to 28 revealed a double peak, the major peak matching the *F. gigantica* nucleotides and the minor peak matching the *F. hepatica* nucleotide across all residues. The consensus sequence is shown at the bottom with the SNPs depicted as arrowheads.

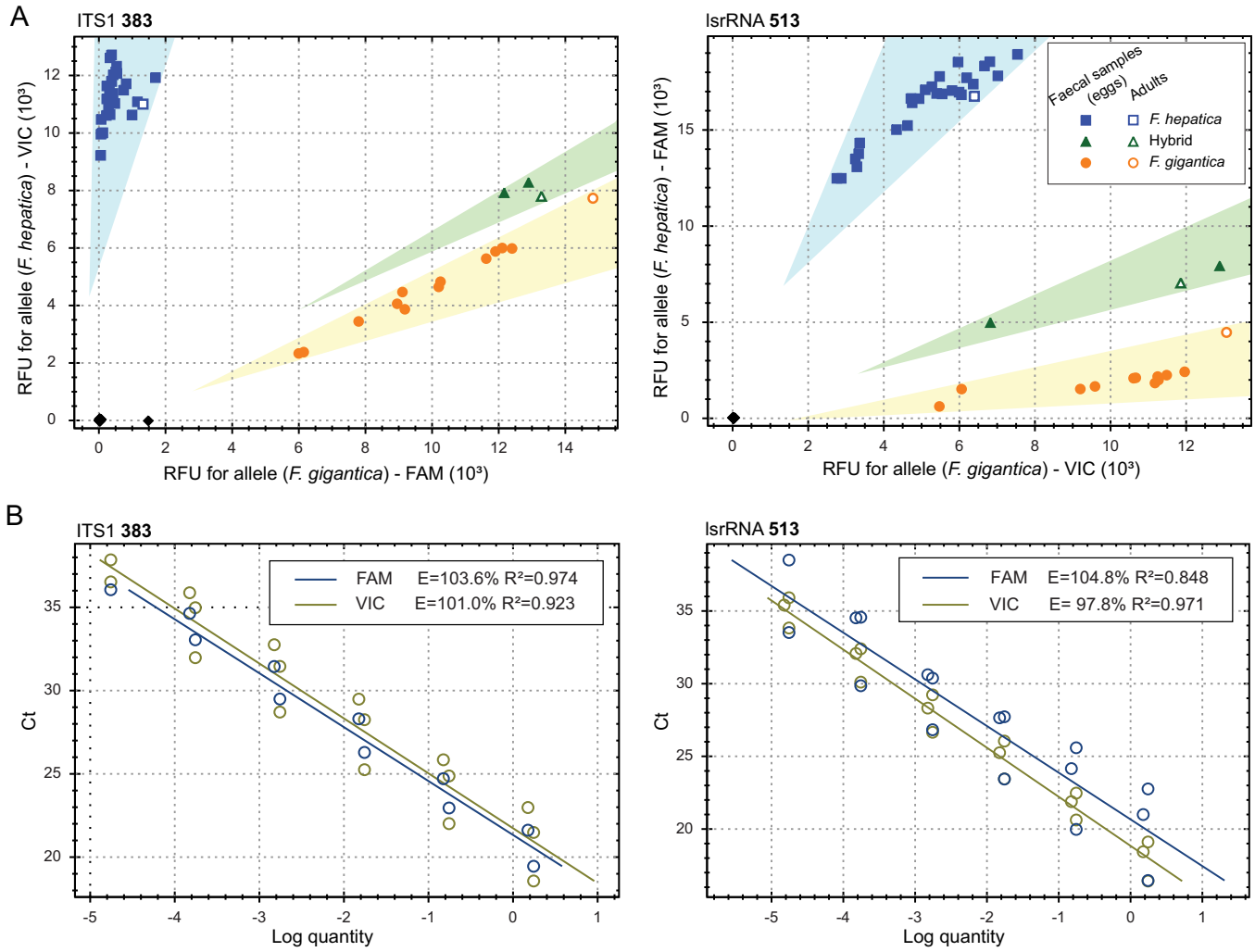

Fig. 3. Analytical sensitivity and specificity of two single nucleotide polymorphism (SNP) assays for the differentiation of *Fasciola hepatica, Fasciola gigantica* and hybrid faecal samples. The analytical specificity (A) of each assay was established via the use of faecal samples from animals known to be *Fasciola*-positive and -negative. *Fasciola hepatica* faecal samples (blue) were sourced from experimentally-infected Merino sheep from a previous study (Calvani et al., 2018) and ranged from 0 to 1043 eggs per gram of faeces (EPG). *Fasciola gigantica* faecal samples (orange) were sourced from infected cattle in Cambodia and ranged from 0.5 to 14 EPG. Hybrid samples (green) were provided by Iwate University, Japan from their laboratory-maintained strains. Negative faecal samples (black) were sourced from a flock of Merino sheep known to be infected with a range of other parasites. Shaded areas indicate the boundaries between the species as defined by the location of the positive controls. DNA extraction controls and no template PCR negative controls are also represented by black squares. The analytical sensitivity (B) of two SNP assays targeting residues 383 and 513 of ITS1 and lsrRNA, respectively, was determined via a standard curve of 10-fold serial dilutions of adult *F. hepatica, F. gigantica* and hybrid DNA using concentrations of 1.75×10^{1} to 1.75×10^{-6} ng/µl. The efficiency of each assay is shown in the top right insets of each standard curve. RFU, relative fluorescence units; FAM, fluorescein; VIC dye.

specific PCR amplicons when used on DNA from faecal samples (LabArchives: Supplementary Data S5) (Marcilla et al., 2002; Itagaki et al., 2005). The substitution of the existing reverse primers for the newly designed reverse primers produced unambiguous amplicons using DNA isolated from *F. hepatica* and *F. gigantica* adults, eggs or directly from *Fasciola*-positive faecal samples of lengths 618 bp and 570 bp for ITS1 and partial lsrRNA, respectively (ITS1: S0762/S0811; lsrRNA: S0756/S0807, Table 1) (Supplementary Data S5).

3.3. TaqMan probe-based SNP genotyping assays enable rapid screening of faecal samples collected in areas of frequent livestock thoroughfare

To test the high-throughput screening capacity of both SNP genotyping assays, we tested a total of 153 faecal samples from local cattle in 27 villages across Northern Laos from an area of frequent livestock movement (Fig. 4A; LabArchives: Supplementary Data S6). Of these, 75 were collected from five villages (15 samples/village) in the 2018 wet season and another 78 were collected randomly from an additional 22 villages during the 2019 dry season. In total 91/153 samples from 21 villages were positive for *Fasciola* spp. (Fig. 4B). According to the SNP genotyping assay targeting residue 513 of the partial lsrRNA, 11/91 samples demonstrated evidence of either *F. hepatica*/*F. gigantica* co-infection or infection with *Fasciola* hybrids, while one sample (1–7) indicated infection with *F. hepatica* only (Fig. 5A). The samples were further tested using the SNP genotyping assay targeting residue 383 of ITS1, as well as with an existing assay for ITS2 (S0754/S0755; ProFh S0770; ProFg S0771, Table 1) (Fig. 5B) (Alasaad et al., 2011). Well-defined separation between the species clusters was observed for the SNP genotyping assay targeting lsrRNA and to a lesser extent the assay targeting ITS1, but the ITS2 assay miscalled sample 1–14 and there was overlap between the *F. gigantica*-positive control and sample 1–2 (Fig. 5A).

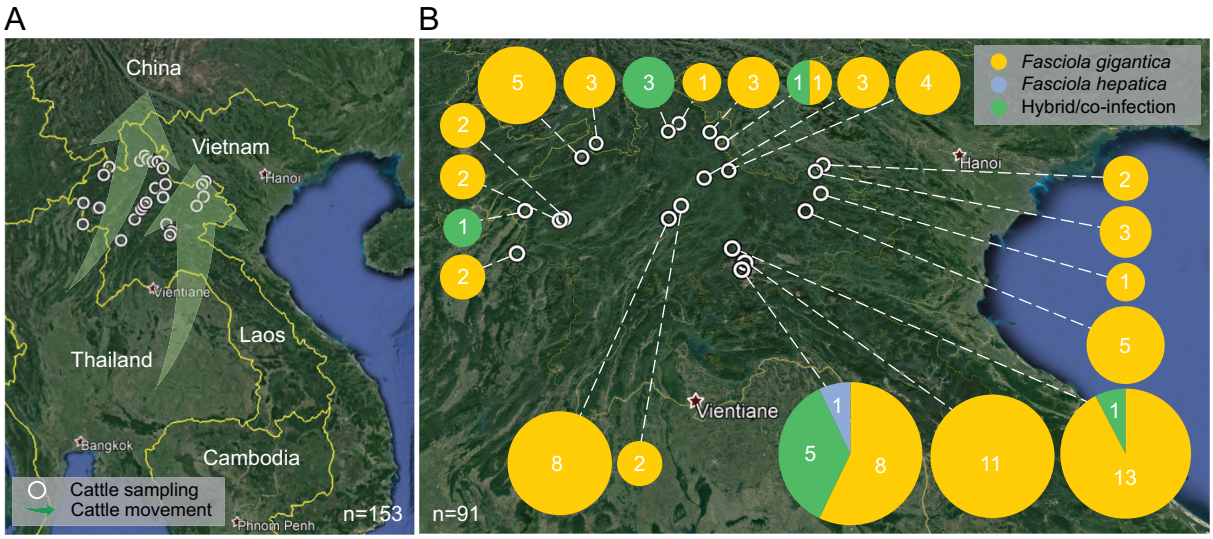

Fig. 4. *Fasciola* spp. detected in faecal samples from local cattle in an area of Southeast Asia with frequent livestock trafficking using a single nucleotide polymorphism assay targeting lsrRNA. Faecal samples were collected from a total of 153 local cattle across 27 villages (indicated by white circles) in Northern Laos, while net international livestock movements are indicated by arrows (A). Of the 27 villages sampled, 21 were positive for *Fasciola* spp. DNA. The proportion of animals determined to be infected with either *Fasciola hepatica* (blue), *Fasciola gigantica* (yellow) or demonstrating *Fasciola* hybrid infection/co-infection of *F. hepatica* and *F. gigantica* (green) for each village is shown (B). Google Earth 7.3.2.5776 (August, 2019) Data SIO, NOAA, U.S. Navy, NGA, GEBCO, Image Landsat/Copernicus, US Department of State Geographer© 2018 Google.

3.4. Optimised conventional PCR assays coupled with Sanger sequencing allow the qualitative demonstration of F. hepatica and F. gigantica DNA in faecal samples

The allelic discrimination plot calls (Fig. 5A) of five *Fasciola* hybrid/co-infected faecal samples (1–1, 1–2, 1–11, 1–12 and 1–14) and the one *F. hepatica*-positive faecal sample (1–7) were investigated using our new conventional PCR assays for the *Fasciola*-specific amplification of DNA from faecal samples for ITS1 and partial lsrRNA, and Sanger sequencing (Table 1, Fig. 5B). Using the optimised ITS1 (S0762/S0811, 615 bp amplicon) and lsrRNA (S0756/S0807, 570 bp amplicon) assays, four and six samples, respectively, produced products of a good enough quality for sequencing (Fig. 5B). The two samples that we were unable to produce ITS1 sequences from had faecal egg counts of five EPG (sample 1–1) and 35 EPG (sample 1–12), while the remaining four samples (1–2, 1–7, 1–11 and 1–14) had faecal egg counts of 7, 8.5, 23.5, and 18.5 EPG, respectively.

The faecal sample (1–7) identified as containing pure *F. hepatica* DNA using the SNP genotyping assays produced a Sanger chromatograph that either demonstrated unambiguous *F. hepatica* residues (forward strand ITS1) or double peaks, the dominant of which matched the *F. hepatica* residue (reverse strand ITS1, forward strand lsrRNA) (Fig. 5B). Four of the faecal samples (1–1, 1–2, 1–11, 1–12) called by the SNP genotyping assays as co-infected/*Fasciola* hybrid infected produced Sanger chromatographs showing double peaks with the dominant component being *F. gigantica* residues analogous to the reference hybrid chromatograph (Fig. 1). The remaining faecal sample (1–14) produced unambiguous *F. gigantica* Sanger chromatographs (Fig. 5B). Amplification of the single copy PEPCK gene was not successful on DNA isolated from eggs in faecal samples (Fig. 2).

3.5. NGS of species-differentiating SNPs gives a quantitative evaluation of F. hepatica and F. gigantica nucleotides in ITS1 and lsrRNA samples

To address the issues of potential co-infection with *F. hepatica* and *F. gigantica* or the presence of *Fasciola* hybrids (*F. hepatica*/*F. gigantica* hybrid) we designed new PCR primer pairs that specifically targeted key ITS1 and lsrRNA SNPs within approximately 200 nt long amplicons (Table 1, Fig. 6A, B). Sequencing using the Illumina NGS platform was used to quantitatively evaluate the contribution of each species to these key SNPs using amplicons in the exponential phase of PCR (Supplementary Data S2). The PCRs were pooled per sample and on average we obtained 99,540 (59,937–121,986) paired reads per sample. Using *F. hepatica* and *F. gigantica* adult DNA, the NGS sequencing confirmed the presence of all dominant nucleotides within ITS1 (461 and 481 according to the sequenced amplicon) and lsrRNA (513 according to the sequenced amplicon) (Fig. 6C, D). The hybrid adult fluke DNA Illumina paired reads revealed that 11% matched ITS1 SNP residues (461, 481) and lsrRNA residue (513) of *F. hepatica* and the remaining (89%) matched *F. gigantica* reads. We then used faecal sample DNA (DNA isolated from *Fasciola* spp. eggs), processed using the method outlined in Section 2.1.2, that the SNP genotyping assays identified as showing both *F. hepatica* and *F. gigantica* amplification profiles (samples 1–7, 1–11). NGS confirmed the presence of residues matching *F. hepatica* and *F. gigantica* in both samples. Sample 1–7 had approximately 3/4 (75%) *F. hepatica* nucleotides across all three key resides, while sample 1–11 had 1/4 (25%) *F. hepatica* residues (Fig. 6C, D). Across all sequenced residues there was negligible (<1%) presence of other nucleotides (LabArchives: Supplementary Data S7). Using the DADA2 pipeline we were able to demonstrate the dominant presence of exact amplicon sequence variants (ASVs) matching the reference genotype for either *F. hepatica* and/or *F gigantica* in the studied samples. SNP analysis was confirmed via the DADA2 approach, with the presence of 100% matching ASVs within adult *Fasciola* spp. DNA. Similarly, DADA2 demonstrated the presence of ~10% of *F. hepatica* and ~90% of *F. gigantica* ASVs from the *Fasciola* hybrid sample (Table 2).

To inquire into the ability of the NGS approach to detect the degree of mixtures of DNA, we manually isolated and mixed *F. hepatica* and *F. gigantica* eggs. We mixed 10, 50 and 90 eggs of one species complemented with the other species up to a total of 100 eggs per sample (1:9; 1:1; 9:1 egg ratios) and isolated DNA as described in Section 2.1.2. The NGS approach detected both *F. hepatica* and *F. gigantica* nucleotides at all three key residues in ITS1 (461, 481) and at the single lsrRNA residue (513) (Fig. 6C, D) as well as ASVs matching reference genotypes of *F. hepatica* and/or *F. gigantica* (Table 2). Nucleotides of *F. hepatica* in the lowest

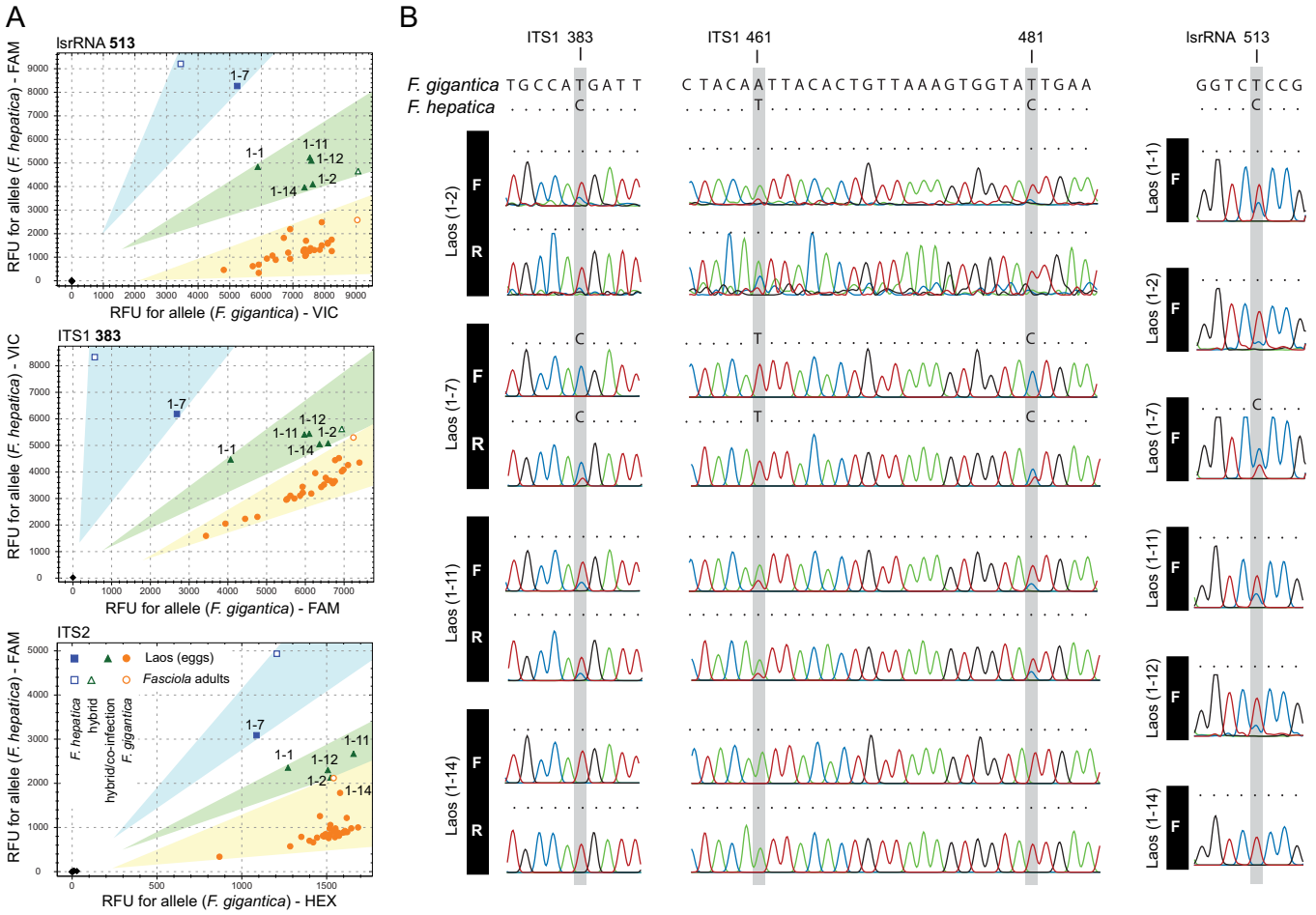

Fig. 5. Single nucleotide polymorphism (SNP) assays for the differentiation of *Fasciola hepatica* and *Fasciola gigantica* DNA in field samples. Allelic discrimination (AD) plots for three SNP assays for a selection of faecal samples from Lao cattle collected during the 2018 dry season with varying egg counts of unknown *Fasciola* spp. identity (A). Clusters are colour-coded according to the positive controls for *F. hepatica* (blue), *F. gigantica* (orange) and *Fasciola* hybrid (green) DNA. The green clusters depict animals infected with either *Fasciola* -hybrid and/or co-infection of *F. hepatica* and *F. gigantica*. The signal for each probe is reported in relative fluorescent units (RFU) and the fluorophores used are VIC, FAM and HEX. The Lao faecal sample clustering with *F. hepatica* (1–7) and those clustering with *Fasciola* hybrid DNA (1–1, 1–2, 1–11, 1–12 and 1–14) are labelled; note that sample 1–14 clustered with *F. gigantica* in the ITS2 assay. The key to the samples and controls is included as an inset in the ITS2 assay AD plot. Black diamonds depict the no-target controls. The sequence alignment of the conventional PCR amplicons for ITS1 and lsrRNA to confirm the *F. hepatica* and hybrid/co-infection AD plot cluster associations is also shown (B). Residues 383, 461 and 481 relative to the ITS1 amplicon, and residue 513 relative to the lsrRNA amplicon are shaded. Reference sequences are depicted above the chromatographs. For ITS1 both of the forward (F) and reverse (R) Sanger sequenced strands are shown; for lsrRNA only the forward (F) strand is shown.

1:9 sample (Fh10:Fg90, 10% *F. hepatica* eggs) were detected at 0.5–1%. Nucleotides of *F. hepatica* in the sample with 1:1 eggs (Fh50:Fg50, 50% of *F. hepatica* eggs) were detected at 3–4%. Nucleotides of *F. hepatica* in the sample with 9:1 eggs (Fh90:Fg10, 90% of *F. hepatica* eggs) were detected at 26–28%. No bands were visible after amplification of PEPCK for any of the mixed egg samples (Fig. 2).

We then scored all sequenced (ITS1, lsrRNA) residues for the highest presence of variants and selected the top three. At ITS1, two of the three were those known variables between *F. hepatica* and *F. gigantica* (461 and 481). The additional ITS1 residue 447 was polymorphic regardless of the sample it came from (Fig. 6C). At lsrRNA, one of the three was the known residue (513) differentiating *F. hepatica* and *F. gigantica*, while the additional two lsrRNA residues (449, 431) were polymorphic regardless of the sample they came from (Fig. 6D).

4. Discussion

The ITS1 and lsrRNA-based SNP genotyping assays developed herein allow wide-scale ante-mortem detection and *Fasciola* spp. differentiation in ruminant faecal samples, while the NGS assays provide a quantitative investigation into the proportion of DNA from each *Fasciola* sp. Combined, these assays were used to identify local cattle harbouring *F. hepatica* DNA in Northern Laos, confirming the suspected establishment of the *F. hepatica* life cycle in the region as a consequence of frequent international livestock movements.

The objective of ante-mortem *Fasciola* spp. identification is dependent on the detection of DNA from *Fasciola* eggs in faecal samples. While a single *F. hepatica* adult is known to shed up to 24,000 eggs per day in sheep, the increased faecal volume of large ruminants contributes to lower *Fasciola* spp. egg counts (often <20 EPG), thereby diluting the templates for detection by PCR amplification (Happich and Boray, 1969b). We elected to use multi-copy DNA markers for ante mortem diagnostics over single copy markers to increase the overall sensitivity of the assays. The rDNA units (including ITS1 and lsrRNA) occur as several hundred tandemly repeated copies (Hillis and Dixon, 1991). To the best of our knowledge, the exact number of rDNA units in *Fasciola* spp. is unknown, although they are assumed to have identical inheritance from both parents due to concerted evolution (Hillis and Dixon, 1991). Our results demonstrate the inferiority of a single-copy nuclear marker (PEPCK, traditionally used for *Fasciola* spp. identifi-

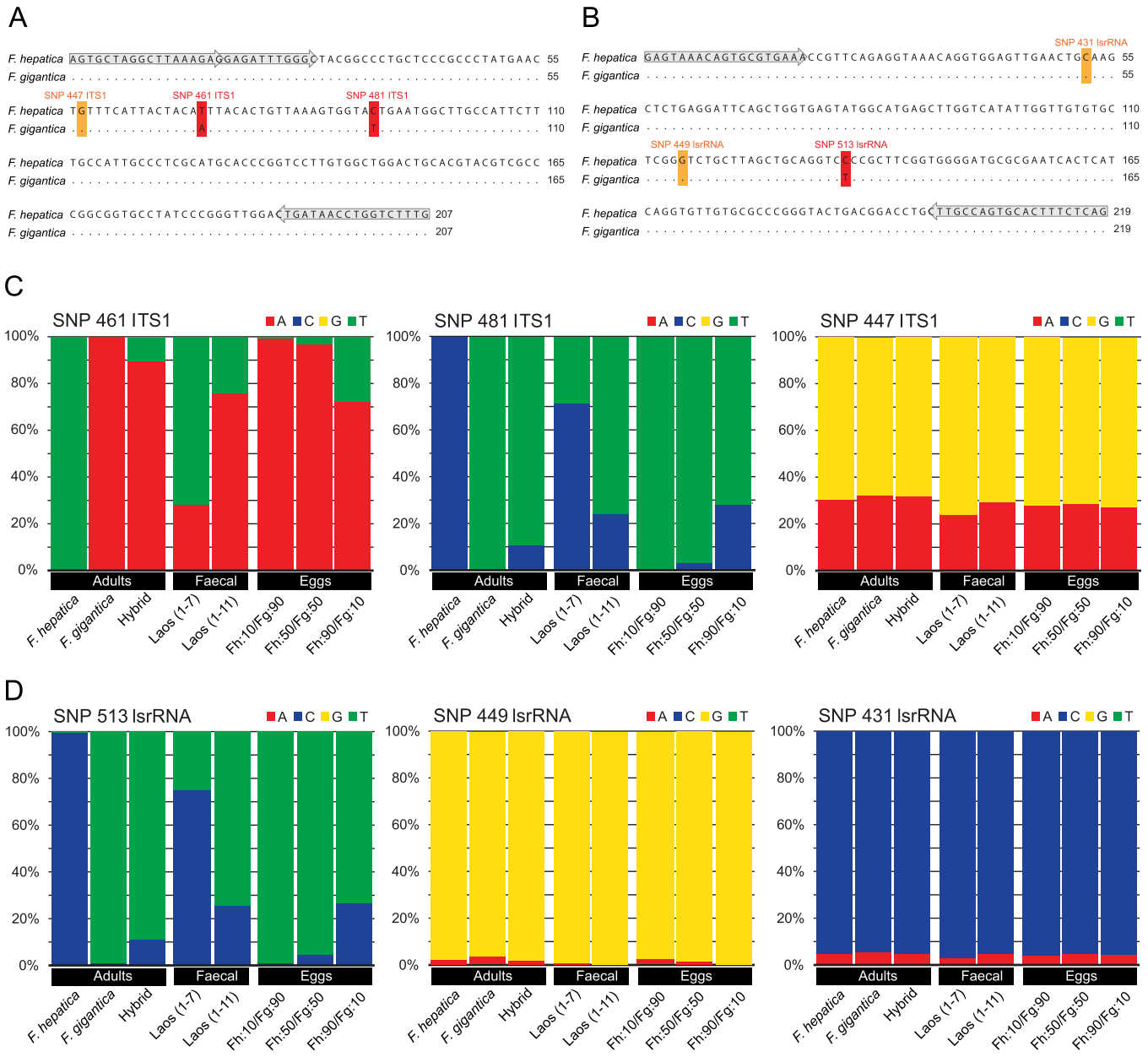

Fig. 6. Single nucleotide polymorphisms (SNPs) quantified using Next Generation Sequencing for the differentiation of *Fasciola hepatica* and *Fasciola gigantica* DNA. Alignments of the amplified ITS1 (A) and lsrRNA (B) sequences used for Illumina DNA sequencing. Identical nucleotides are indicated by '.' and the amplification primer sequences are highlighted grey. Known differentiating SNPs between *F. hepatica* and *F. gigantica* are in red and those identified as polymorphic regardless of species are in yellow. The proportion of nucleotides across the three most variable nucleotides using Illumina DNA Next Generation Sequencing reads within the ITS1 (C) and lsrRNA (D) amplicon are shown. The PCRs for each target were pooled per sample and on average we obtained 99,540 (59,937–121,986) paired reads per sample. Samples from adult flukes belonging to *F. hepatica* from Australia, *F. gigantica* from Laos and *Fasciola* hybrid DNA from Japan served as controls. Faecal samples containing *Fasciola* spp. eggs were from the Laos survey (1–7; 1–11) and demonstrate the mixed presence of both *F. hepatica* (Fh)/*F. gigantica* (Fg) DNA that could either represent the presence of hybrid specimens or mixed infection. A mixed egg infection was mimicked using samples with eggs belonging to both species at ratios of 1:9 (Fh:10/Fg:90), 1:1 (Fh:50/Fg:50) and 9:1 (Fh:90/Fg:10).

cation) for the ante-mortem detection of DNA in faeces, where we were unable to detect DNA from three 100 egg replicates (Shoriki et al., 2016). One alternative could have been to use mitochondrial DNA (mtDNA) markers that also exist in multiple copies in *Fasciola* spp. (Threadgold and Read, 1968; Tielens, 1994). The mode of inheritance of mtDNA in hermaphroditic *Fasciola* spp., however, is not known and so the use of these markers may bias species differentiation, especially considering the existence of *Fasciola* hybrids (Itagaki et al., 2011). Using both rDNA markers (ITS1, lsrRNA) we were able to reliably detect *Fasciola* spp. DNA in faecal samples regardless of the faecal egg counts.

During validation of the SNP genotyping and NGS assays we observed consistently better amplification of *F. gigantica* over *F. hepatica,* irrespective of the residue targeted. The discrepancy was quantified using NGS to detect DNA from different ratios of *F. hepatica* and *F. gigantica* eggs, where mixtures containing 10, 50 and 90% *F. hepatica* eggs demonstrated only 1, 3 and 27% *F. hepatica* residues, respectively, regardless of the rDNA target. Considering that the PCR chemistry and DNA templates were consistent across all samples, reactions and loci, our results imply that there may be different copy numbers of rDNA units in each species, with *F. gigantica* containing the higher number (Polz and

Table 2
Summary of *Fasciola* spp. exact amplicon sequence variants using the DADA2 pipeline.

	F. hepatica	*F. gigantica*	*Fasciola* hybrid	Fh:10/Fg:90	Fh:50/Fg:50	Fh:90/Fg:10	Laos (1–7)	Laos (1–11)
Total (ITS1 rDNA)	79,412	64,220	56,593	69,065	80,041	51,566	48,055	69,711
F. hepatica (reference genotype)	78,875 (100%)	0 (0%)	5,690 (10.1%)	0 (0%)	2,419 (3.0%)	14,286 (28.0%)	34,584 (72.9%)	16,488 (23.9%)
F. gigantica (reference genotype)	0 (0%)	63,614 (100%)	50,582 (89.9%)	68,611 (100%)	77,223 (97.0%)	36,786 (72.0%)	12,857 (27.1%)	52,655 (76.1%)
F. hepatica (all genotypes)	79,412 (100%)	222 (0.4%)	5,896 (10.4%)	219 (0.3%)	2,592 (3.3%)	14,427 (28.0%)	34,753 (72.3%)	16,714 (24.0%)
F. gigantica (all genotypes)	0 (%)	63,998 (99.6%)	50,697 (89.6%)	68,846 (99.7%)	77,449 (96.7%)	37,139 (72.0%)	13,302 (27.7%)	52,997 (76.0%)
Total (lsrRNA)	37,103	30,208	24,939	21,291	34,863	41,975	8,811	40,761
F. hepatica (reference genotype)	35,512 (100%)	0 (0%)	2,549 (10.3%)	99 (0.5%)	1,197 (0.4%)	10,766 (26.8%)	6625 (75.2%)	9,887 (25.2%)
F. gigantica (reference genotype)	0 (0%)	30,028 (100%)	22,179 (89.7%)	21,116 (99.5%)	33,016 (96.6%)	29,432 (73.2%)	2,186 (24.8%)	29,402 (74.8%)
F. hepatica (all genotypes)	36,919 (99.5%)	180 (0.6%)	2,613 (10.5%)	175 (0.8%)	1,374 (3.9%)	10,786 (25.7%)	6,625 (75.2%)	10,039 (24.6%)
F. gigantica (all genotypes)	184 (0.5%)	30,028 (99.4%)	22,326 (89.5%)	21,116 (99.2%)	33,489 (96.1%)	31,189 (74.3%)	2,186 (24.8%)	30,722 (75.4%)

"reference genotype" is the amplicon sequence variant that had 100% similarity to the *Fasciola hepatica* and *Fasciola gigantica* reference sequences; "all genotypes" had >90% identity to the *F. hepatica* and *F. gigantica* reference sequences (ITS1: AB514849 and KF543340, lsrRNA: AY222244 and AJ439739).

Cavanaugh, 1998). Investigation into the copy number of rRNA genes in *F. hepatica* versus *F. gigantica* is needed to confirm these results. Regardless of the over-representation of *F. gigantica* rDNA units by a factor of 5–10, we were able to detect and differentiate *Fasciola* spp. using rDNA markers.

In the current study, NGS proved superior to traditional Sanger sequencing for the discrimination of *F. hepatica* from *F. gigantica* DNA in mixed samples and for the identification of *Fasciola* hybrid adults. This is largely due to the analysis of the proportion of SNPs in individual Illumina reads via NGS versus the interpretation of consensus sequences of all nucleotides in Sanger chromatographs, and is analogous to other NGS assays such as one recently developed for the detection of benzimidazole resistance in trichostrongylids of sheep (Avramenko et al., 2019). Regardless of how the end product is analysed, both methods rely on the generation of PCR amplicons that reliably and proportionally represent the DNA template (Polz and Cavanaugh, 1998). We submitted samples for Sanger sequencing at both the exponential and plateau phase of the reaction, confirming the impacts of template saturation on *Fasciola* hybrid amplicons. Saturation bias was then specifically addressed by optimising our NGS reactions to ensure that the PCR product was in the plateau phase prior to sequencing. These results are significant and suggest that the traditional use of Sanger sequencing for *Fasciola* spp. identification may be responsible for the misidentification of *Fasciola* spp. and a subsequent underrepresentation of *Fasciola* hybrids in the literature.

When used to screen 153 local cattle in Northern Laos for *Fasciola* spp. infection, use of the above assays allowed the identification of 12 animals harbouring *F. hepatica* DNA. *Fasciola hepatica* is currently considered exotic to Laos, despite DNA being detected in neighbouring countries via the identification of hybrid/introgressed forms. Due to the need for an aquatic snail intermediate host, the *F. hepatica* life cycle must be established in the region for local animals to have become infected (Bui et al., 2016; Le et al., 2008; Wannasan et al., 2014; Salzer and Schmiedel, 2015). Our findings imply that one or more snail species capable of infection with *F. hepatica* is present in Northern Laos, and suggests that translocation of this parasite may have occurred via the importation of infected animals from *F. hepatica*-endemic regions. This theory is supported by the frequent importation of livestock into the region from countries such as Brazil, Argentina and Australia, and suggests that *F. hepatica* should be considered a potential pathogen of both human and animal concern in Southeast Asia (USDA, 2019).

Molecular tools for the diagnosis and species differentiation of parasites are becoming increasingly common, yet despite their significant human and animal health impacts, *Fasciola* spp. are often overlooked (Avramenko et al., 2015, 2017; Papaiakovou et al., 2019). By enabling *Fasciola* spp. differentiation from faecal samples, the ante-mortem assays designed in the current study extend our ability to investigate the distribution of both species of this parasite beyond the abattoir, providing the foundation for future wide-scale epidemiological investigations (Sothoeun et al., 2006; da Costa et al., 2019). We have proven the superiority of our NGS assay over traditional Sanger sequencing for species identification from *Fasciola* adults and faecal samples containing mixed DNA due to the ability to provide quantitative resolution of key residues, and recommend their use for species identification hereafter. Finally, we have identified the presence of *F. hepatica* DNA in local cattle in Northern Laos, an area of frequent international livestock movements, suggesting the establishment of the life cycle in the region.

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Chapter 6

General discussion, conclusion and future directions

Fasciolosis in developing countries – why we should care

In developed countries, such as Australia and the United Kingdom, fasciolosis due to infection with *Fasciola hepatica* is often acknowledged as a significant cause of livestock production losses (Charlier et al., 2014; Howell et al., 2015; Beesley et al., 2018; Kelley et al., 2020). However, infection of livestock with *Fasciola gigantica* in developing countries is often ignored. Such is the case in Southeast Asia where the combination of aquatic rice production and large ruminant husbandry provides the ideal habitat for parasite proliferation (ACIAR, 2008). Production impacts due to infection with either *Fasciola* species range from sudden death, in the case of acute infections, to chronic losses due to decreased milk yield and fertility, reduced body condition scores, liveweight gains and wool growth (Hope Cawdery, 1976; Hope Cawdery et al., 1977; Charlier et al., 2014; Howell et al., 2015; Köstenberger et al., 2017; da Costa et al., 2019). Fasciolosis has been shown to modulate the immune response of infected hosts, leaving them more susceptible to infection with other pathogens and confounding the outcomes of their diagnostic tests (Brady et al., 1999; Jen et al., 2012; Naranjo Lucena et al., 2017). While country-specific data is lacking, the global economic impact of fasciolosis is estimated to exceed US $3 billion/year (Keiser and Utzinger, 2005; Torgerson and Macpherson, 2011). These production impacts and associated economic losses make *F. hepatica* one of the primary focusses of integrated parasite management programs in developed countries (Charlier et al., 2014; Neeson and Love, 2014). Importantly, the diagnosis and control of fasciolosis in the low-input agricultural systems prevalent throughout Southeast Asia are often overshadowed by the more apparent viral and bacterial constraints to production such as Foot and Mouth Disease and Haemorrhagic Septicaemia (Rast et al., 2013; Rast et al., 2014; Young et al., 2014).

While the production impacts of fasciolosis are of concern in their own right, the potential human health impacts are the most often overlooked (Le et al., 2008; Bless et al., 2015). Both *F. hepatica* and *F. gigantica* are zoonotic and highly pathogenic, with ectopic and aberrant fasciolosis further complicating disease outcomes and management (Arjona et al., 1995; Mas-Coma et al., 2014). As a result of the increased number of human infections diagnosed in Europe in the 1990s and early 2000s, growing concerns about human fasciolosis initiated an investigation by the World Health Organisation (WHO) (Mas-Coma et al., 1999). Despite delivering information regarding the epidemiology, distribution and impacts of human fasciolosis, this WHO investigation failed to generate the necessary motivation to address this disease in regions where it is the most neglected. That is, in the least developed countries where livestock production and human sanitation practices provide an ideal scenario for high levels of both human and animal infection.

Accelerating the global distribution of *Fasciola* spp. via international livestock movements – global oversight of human-mediated parasite translocation

Further complicating the issue of fasciolosis in Southeast Asia in particular is the potential introduction of *F. hepatica*-infected livestock as a result of the increased demand for animal-derived protein by the rapidly-expanding middle classes of China and Vietnam (Smith et al., 2015; USDA, 2019). This increased protein demand has largely been met by the importation of animals from *F. hepatica*-endemic countries including Australia, Argentina and Brazil (USDA, 2019). Translocation of *F. hepatica* into the *Fasciola*-free region of Western Australia has historically been prevented via the implementation of a strict treatment and monitoring program (Palmer et al., 2014; Twentyman, 2018; Vassallo, 2019). However, no such requirements exist for the export of animals from endemic regions into international livestock markets.

While the global distribution of *Fasciola* spp. has long been associated with post-domestication livestock movements, the role that the international trade of livestock plays in accelerating the translocation of these parasites from endemic to non-endemic areas has, for the most part, been ignored (Mas-Coma et al., 2009). The post-mortem examination of livers in Saudi Arabian abattoirs has demonstrated the capacity of the international livestock trade to facilitate the movement of these parasites via the detection of *Fasciola* spp. in 10-22% of imported sheep (Sanad and Al-Megrin, 2005; Shalaby et al., 2013). The morphological and molecular characterisation of the adult flukes collected in one of these studies revealed the importation of not only *F. hepatica* and *F. gigantica*, but also an 'intermediate' *Fasciola* species identified by mixed RAPD-PCR banding patterns and ITS-1 nucleotide sequences (Shalaby et al., 2013). By carrying the genetic signatures of both species, this 'intermediate' *Fasciola* spp. is assumed to be the outcome of interspecific hybridisation between *F. hepatica* and *F. gigantica.*

Fasciola hepatica and *Fasciola gigantica* hybridisation – just a fluke or something more?

Hybridisation is reported to occur in areas of *Fasciola* spp. sympatry as the result of interspecific mating between *F. hepatica* and *F. gigantica* (Agatsuma et al., 2000; Le et al., 2008; Nguyen et al., 2012; Ichikawa-Seki et al., 2017; Saijuntha et al., 2018). Experimentally, successful hybridisation between these parasites has been demonstrated via co-infection of Wistar rats and morphological and molecular characterisation of the resulting F1 and F2 generations (Figure 1) (Itagaki et al., 2011). The experimental *Fasciola*-hybrid adults demonstrate an intermediate body-length to body-width ratio between that of their parent species and appear to be more infectious than *F. gigantica* alone based on higher than previously reported recovery rates. Hybridisation does not necessarily generate permanent genetic change, however, and the intermediate phenotypes observed in the experimental *Fasciola*-hybrids may be inconsequential in the long term due to the limited viability of the successive generations (Itagaki et al., 2011; Harrison and Larson, 2014).

While successful hybridisation between *F. hepatica* and *F. gigantica* has been demonstrated experimentally, there is no empirical evidence supporting introgression between these two species (Figure 1) (Itagaki et al., 2011). The terms 'hybridisation' and 'introgression' are used without differentiation throughout the *Fasciola* spp. literature and often without any consideration of the functional and epidemiological implications of either state (Agatsuma et al., 2000; Le et al., 2008; Saijuntha et al., 2018). Introgression is the transfer of genetic material from one species into the genome of another, usually by the repeated backcrossing of hybrids with either of the original parental species (Figure 1) (Harrison and Larson, 2014; Mallet et al., 2016). The observed limited viability of *Fasciola*-hybrids tends to suggest that introgression between these two parasites is unlikely (Itagaki et al., 2011). However, the longevity of *Fasciola* spp. adults within their definitive hosts means that even a single hybrid generation may provide ample opportunity for introgression in co-infected animals (Durbin, 1952). In some cases, such as in the hybridisation of *Helianthus* sunflower species, a reduction in the fertility of F1 and F2 generations does not present an insurmountable barrier for the establishment of new lineages (Heiser, 1947; Heiser et al., 1969; Rieseberg, 1991). Instead, this temporary bottleneck can be overcome by the frequency with which opportunities for hybridisation and introgression occur (Arnold and Hodges, 1995).

The current lack of differentiation between hybridisation and introgression in *Fasciola* spp. is alarming given the phenotypic and epidemiological outcomes of introgression observed in other species. Adaptive introgression describes the establishment of beneficial alleles within a population and has the potential to drive evolutionary changes at a much faster rate than would normally occur by mutation alone (Figure 1) (Mawejje et al., 2013; Mallet et al., 2016). Adaptive introgression has seen the transfer of resistance genes against organophosphates and pyrethrum between *Anopheles* mosquito species, as well as the induction of ivermectin resistance in lab-maintained *Haemonchus contortus* populations (Weill et al., 2000; Djogbenou et al., 2008; Redman et al., 2012; Norris et al., 2015). Thus it is not inconceivable that adaptive introgression may result in the transfer of anthelmintic resistance in *Fasciola* spp. due to the importation of triclabendazole-resistant *F. hepatica* into *F. gigantica*-endemic regions (Figure 1) (Overend and Bowen, 1995; Brockwell et al., 2014; Kelley et al., 2020). Introgression between these two species may have other important human and animal health impacts, including increased infectivity, virulence and pathophysiology, not to mention any potential implications surrounding the infection of intermediate hosts and survival of eggs and metacercariae in the environment. Considering how little is known about introgression in these parasites, the increased likelihood of co-infection and hybridisation as a result of the international trade of infected livestock is particularly concerning.

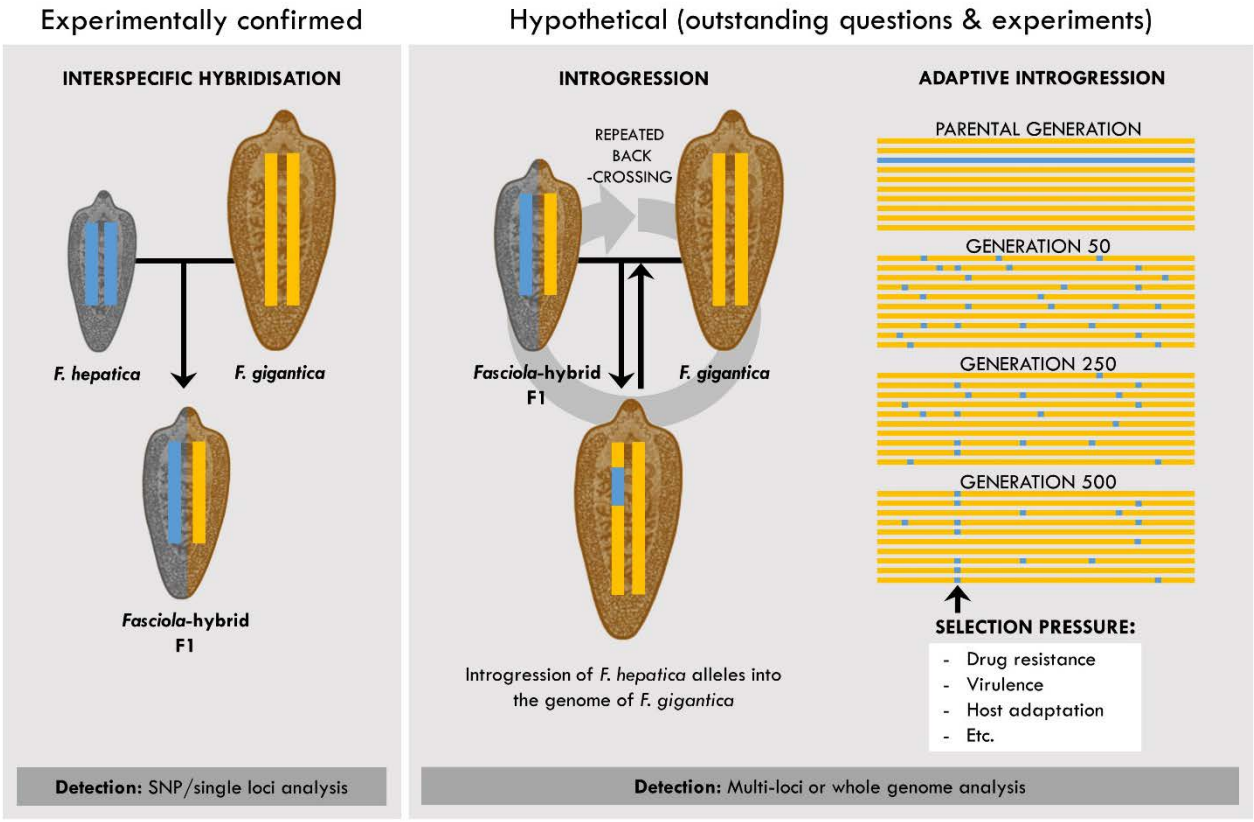

Figure 1. Hybridisation and introgression between *Fasciola hepatica* and *Fasciola gigantica* and the influence of possible selection pressures. Hybridisation has been experimentally demonstrated as a result of interspecific mating between *F. hepatica* and *F. gigantica*, with the F1 generations exhibiting an intermediate body-width to body-length ratio between the two species as shown (left). Introgression is the transfer of genetic material from one species into the genome of another, usually as a result of backcrossing of hybrids with either of the original parental species. The occurrence of introgression between *Fasciola* spp. remains unproven, but has been theoretically represented here as a result of the repeated back-crossing of *Fasciola*-hybrids with *F. gigantica* (centre). Adaptive introgression is the establishment of beneficial alleles within a population and may occur as a result of various forms of selection pressure. The thick lines in the adult flukes are representative of the nuclear genomes of each species with blue indicating *Fasciola hepatica* and yellow indicating *Fasciola gigantica*. The two colours in the *Fasciola*-hybrid adult is a theoretical representation of F1 generations as a result of hybridisation between both parental species. A simplified diagram demonstrating the potential random drift of introgressed alleles from *F. hepatica* (blue) into the genome of *F. gigantica* (yellow) and the fixation of new alleles at a single locus as one outcome of selection pressure is shown (right). Initially, many alleles introgress, although incompatible alleles are eventually selected against and disappear. By generation 500, the advantageous allele is moving towards fixation, while other variants persist at a lower rate of recombination. The source of selection pressure is population-dependent but may involve adaptations for drug resistance, increased virulence, adaptation to new definitive or intermediate hosts, higher metacercarial output from intermediate hosts, increased temperature tolerance of eggs and/or metacercariae, etc. The demonstration of introgression within a given population is a simplified adaptation of a figure by Martin and Jiggins (2017).

Differentiating hybridisation and introgression in *Fasciola* spp.

The differentiation between hybridisation and introgression is far from trivial in *Fasciola* spp., where their hermaphroditic nature, capacity for parthenogenesis, and the occurrence of polyploids complicate our understanding of these events (Terasaki et al., 2000; Fletcher et al., 2004; Itagaki et al., 2009; Detwiler and Criscione, 2010). However, the major ambiguity surrounding the distinction of hybridisation from introgression in *Fasciola* spp. is due to a tendency to investigate single loci when a multi-loci or whole-genome approach is required (Figure 2) (Huang et al., 2004; Detwiler and Criscione, 2010; Alasaad et al., 2011; Choe et al., 2011; Martin and Jiggins, 2017; Saijuntha et al., 2018). Most studies have relied on repetitive rDNA units to demonstrate hybridisation and only recently has a nuclear-encoded single copy gene, PEPCK, been investigated (Shoriki et al., 2016; Hayashi et al., 2018). Furthermore, the use of mtDNA alone as evidence for hybridisation in *Fasciola* spp. may be misleading due to a total lack of information concerning mitochondrial inheritance in these parasites and the limited recombination of mitochondrial genomes (Detwiler and Criscione, 2010; Ai et al., 2011b; Moazeni et al., 2012; Liu et al., 2014). Thus, it quickly becomes clear that without knowing more about the genetic makeup of these forms, over-interpretation of naturally-occurring hybridisation and introgression events should be avoided. Experimental back-crossing of *Fasciola*-hybrids with both parental species is needed to aid in the unambiguous confirmation of the occurrence of introgression in these parasites and to provide insights into appropriate markers going forward. Until then, the characterisation of these forms in hybrid zones should involve the comparison of many markers from a pool of individuals to external populations where only a single species is present (Agatsuma et al., 2000).

Identifying areas of parasite sympatry - surveillance of *F. hepatica* and *F. gigantica* infection beyond the abattoir

Prior to further investigations into the true occurrence and impacts of hybridisation and introgression between *F. hepatica* and *F. gigantica*, areas of parasite sympatry providing environments conducive to these events must be identified. Despite a range of methods available for the diagnosis of *Fasciola* spp. infection in livestock, species identification and differentiation was previously confined to the use of adult specimens collected post-mortem (Shalaby et al., 2011; El-Rahimy et al., 2012; Nguyen et al., 2012; Aryaeipour et al., 2017). The morphological identification of *Fasciola* spp. adults and their eggs is unreliable in areas of parasite sympatry, especially given that host species, nutritional, production and health status may result in overlapping egg morphology (Valero et al., 2009). The differentiation of both adults and eggs based on morphology alone is time consuming and requires highly-trained technical personnel, limiting the application of traditional methods in a practical diagnostic setting.

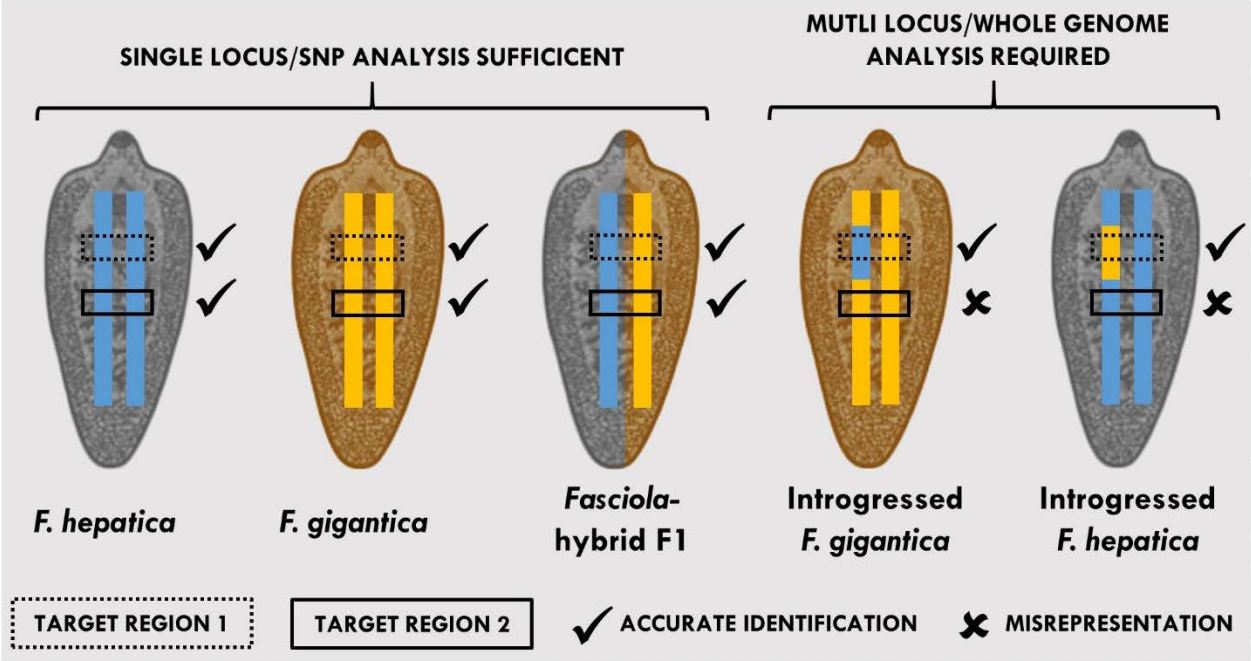

Figure 2. A schematic representation of the limitations of single-locus methods for differentiation between *F. hepatica* and *F. gigantica* adults, their hybrid and introgressed forms using rDNA compared to multi locus and/or whole genome methods. Single targets (indicated by the dotted boxes) may not be appropriate for the identification of introgressed individuals unless previously identified as markers susceptible to introgression. In both of the pure species and in the *Fasciola*-hybrid, accurate identification can theoretically be conducted regardless of the locus targeted. However, in introgressed specimens, these results may misrepresent the true status of the specimen. Ticks are used to indicate regions that have been accurately identified, while crosses indicate misidentification due to the selection of inappropriate markers that have not undergone introgression.

A previous lack of ante-mortem tools for *Fasciola* spp. differentiation in mammalian hosts restricted our ability to monitor potential parasite translocation and hybridisation events to abattoir surveillance alone (Sothoeun et al., 2006; Nguyen et al., 2012; Shalaby et al., 2013). While abattoir surveillance provides important insights into the prevalence and seasonality of infection, gaining access to these sites can be difficult, particularly in developing countries. In these areas, limited animal traceability further hinders a deeper understanding of fasciolosis epidemiology. Ante-mortem *Fasciola* spp. differentiation extends our ability to investigate the distribution of each species beyond the abattoir, helping to identify infection sources and highlight international parasite movements, and extending our knowledge of potential hybridisation zones by identifying areas of parasite sympatry.

Cracking eggs to unlock ante-mortem molecular diagnosis of fasciolosis

Species differentiation prior to completion of the pre-patent period (PPP) using molecular methods is inherently prohibited by the localisation of the immature stages within the liver of infected hosts. Once mature flukes commence egg-laying, the faeces of infected hosts become an abundant and non-invasive source of genetic material. The molecular differentiation of *Fasciola* spp. in live animals has previously been restricted by a perceived lack of genetic material available for molecular characterisation (Ai et al., 2011a). Historically, molecular methods for *Fasciola* spp. identification had avoided the use of eggs in faeces either due to the apparent difficulty in rupturing their robust outer shells, preventing access to the genetic material within, or because of the presence of faecal inhibitors limiting downstream applications (Arifin et al., 2016). In this thesis the dogma surrounding the disruption of the tough outer shell of these eggs was challenged and resolved via the use of a high-speed benchtop homogeniser (**Chapter 2**) (Ai et al., 2010; Calvani et al., 2017). Commercially-available DNA isolation kits specific for use on faecal samples limit the impact of faecal inhibitors, removing the roadblocks preventing the development of molecular diagnostics for ante-mortem *Fasciola* spp. differentiation (Maksimov et al., 2017).

Maximising the analytical sensitivity of diagnostic tests is particularly relevant for the diagnosis of fasciolosis in large ruminants. Cattle typically harbour infections with fewer flukes than sheep, with *Fasciola* spp. eggs further diluted by the increased faecal volume produced by large ruminants (Boray, 1969; Happich and Boray, 1969; Gonzalez-Lanza et al., 1989). The molecular diagnostic workflow presented in this thesis utilised a traditional sedimentation method to concentrate *Fasciola* spp. eggs, allowing the isolation of DNA from a much larger starting volume than is typically used in standard extraction protocols (6 g vs. 150 mg) (**Chapter 2**) (Calvani et al., 2017). This approach provided the desired analytical sensitivity of less than one egg per gram of faeces (EPG) and a diagnostic sensitivity better than traditional sedimentation methods (**Chapter 2**) (Calvani et al., 2017). The limit of detection in 150 mg raw, un-concentrated faeces was examined to provide a more streamlined sample preparation workflow for when greater sample throughput is required (**Chapter 4**) (Calvani et al., 2018a). The results demonstrated a limit of detection of 10 and 20 EPG for sheep and cattle, respectively, which is considered representative of infection with a single fluke (**Chapter 4**) (Happich and Boray, 1969; Calvani et al., 2018a). The use of these molecular diagnostic workflows to diagnose *F. hepatica* in ten experimentally-infected Merino sheep confirmed that the point of first detection was related to the appearance of eggs in faeces and is therefore limited by the PPP (**Chapter 3**) (Calvani et al., 2018b). While it is yet to be seen how long it takes post-treatment for animals to be considered negative using this approach, it may be assumed that the results are dependent on egg clearance.

The differentiation of *Fasciola* spp. in faecal samples – who's who in the poo?

Although the development of a highly-sensitive workflow for the detection of *Fasciola* spp. DNA in eggs enables ante-mortem molecular diagnosis, *Fasciola* spp. differentiation using faecal samples presents its own challenges. Previously, primer sets had been designed for the amplification of pure DNA from adult specimens, resulting in non-specific amplification when applied to faecal samples (Marcilla et al., 2002; Itagaki et al., 2005) (**Chapter 5**). The suite of conventional PCR primers presented herein were designed for the specific amplification of *Fasciola* spp. DNA in more complex sample types. However, the subjective nature of traditional Sanger sequencing and the preferential amplification of one *Fasciola* species over the other was subsequently revealed via a reduction in the number of PCR cycles employed (**Chapter 5**). By taking the impact of cycle numbers and their effect on template saturation into account, this thesis presents thoroughly-validated alternative methods for the molecular identification of *Fasciola* spp., including the first application of Next Generation Sequencing (NGS) to these parasites. The outcome is a selection of tools for both the qualitative and quantitative identification of *Fasciola* spp. in ruminant faecal samples, which enable the wide-scale screening of individual animals while also providing the means for the deeper interrogation of individual parasite specimens and their eggs (**Chapter 5**).

When considered in the context of parasite sympatry, the limitation of molecular methods for species differentiation from faecal samples becomes apparent. Specifically, it is clear that the isolation of DNA from many eggs within an individual sample makes it impossible to differentiate between co-infected animals and those harbouring infections with *Fasciola*-hybrids (Figure 3). Newer molecular tools, such as the TaqMan probe and NGS assays presented herein, enables higher resolution of these ambiguous states but does not resolve them completely (**Chapter 5**). Instead, they provide the foundation for further investigation via highlighting areas of parasite sympatry and/or hybridisation.

Evidence for the translocation of *F. hepatica* into Southeast Asia via international livestock movements: the application of ante-mortem molecular diagnostic tools for wide-scale animal surveillance in Northern Laos

The final outcome of this thesis is the demonstration of *F. hepatica* DNA in an area of Southeast Asia with frequent international livestock movements (**Chapter 5**). The ante-mortem molecular diagnostic tools presented herein were used to screen 153 local cattle in 27 villages across Northern Laos. Of these samples, 91 were PCR positive across 21 villages and *F. hepatica* DNA was detected in 11 animals (**Chapter 5**). Interestingly, all of the villages with animals exhibiting evidence of infection of *Fasciola*-hybrids or *F. hepatica/F. gigantica* co-infection were distributed either along the borders with China and Thailand, or were concentrated around an international Angus beef cattle farm. Although political and cultural reasons prohibited access to abattoir samples to help validate these findings, the detection of *F.*

hepatica DNA in local cattle suggests the establishment of the *F. hepatica* life cycle in Northern Laos via the ingestion of *F. hepatica* or *Fasciola*-hybrid metacercariae and thus the availability of a suitable intermediate snail host. Unfortunately, an investigation of the intermediate snail hosts found in these villages was beyond the scope of this thesis. Regardless, these findings suggest the translocation of *F. hepatica* from endemic regions into Southeast Asia via the international livestock trade, raising concerns about the implications of these parasite movements for future hybridisation and introgression events.

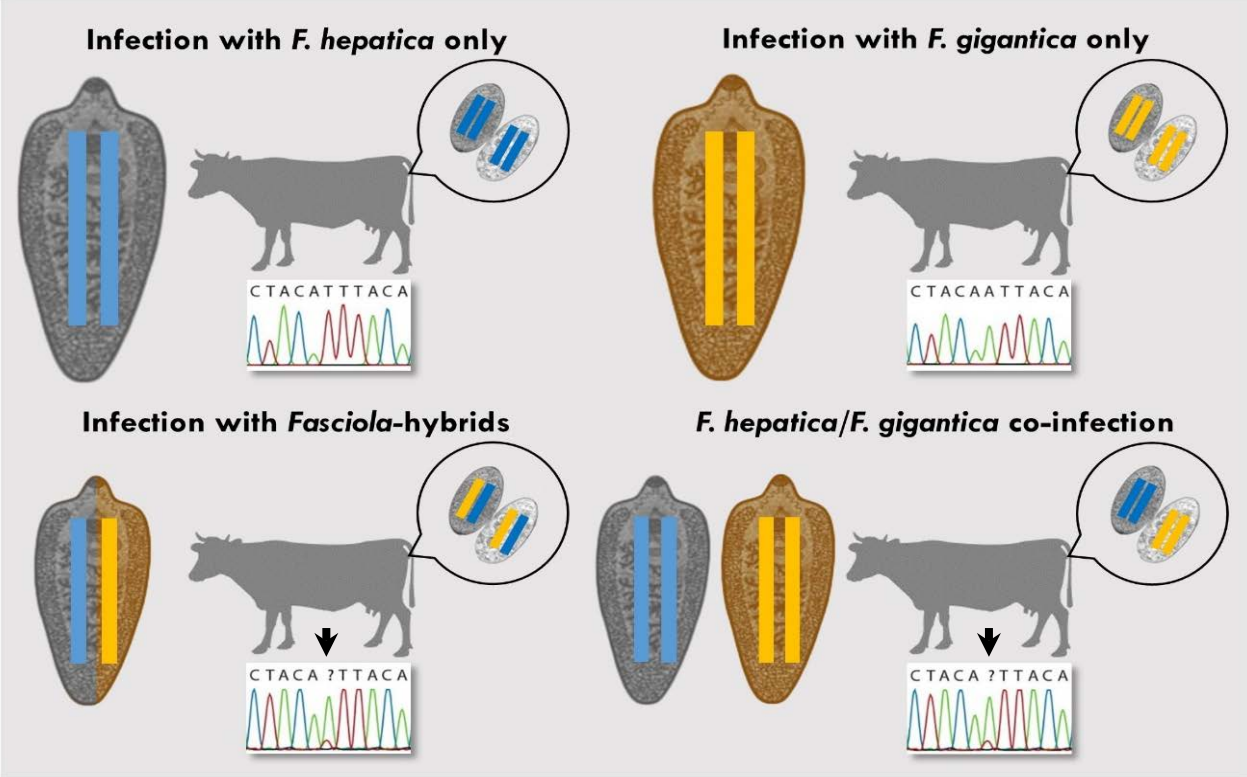

Figure 3. Ante-mortem differentiation of *F. hepatica* and *F. gigantica* infection from co-infection/infection with *Fasciola*-hybrids. Isolation of DNA from eggs in faecal samples does not allow for ante-mortem differentiation of *Fasciola*-hybrid and *F. hepatica*/*F. gigantica* co-infected animals in areas of parasite sympatry. Eggs from faecal samples are represented by the bubbles emerging from the individual animals. The thick lines in the adult flukes and their eggs are representative of the genomes of each species with blue indicating *F. hepatica* and yellow indicating *F. gigantica*. The two colours in the *Fasciola*-hybrid adult and eggs is a theoretical representation of F1 generations as a result of hybridisation between both parental species. The question mark in the chromatograms indicates ambiguous nucleotides from both parental species present when DNA is isolated and sequenced via traditional Sanger sequencing from multiple eggs and is highlighted in both instances by the black arrows. Note that the chromatograms from *Fasciola*-hybrid and *F. hepatica/F. gigantica* co-infected animals are identical and do not allow the differentiation of these two states.

Conclusion and future directions

Given the significant human and animal health impacts of fasciolosis, and that little is known about hybridisation and introgression in *Fasciola* spp., it is concerning that the accelerated global translocation of *F. hepatica* via international livestock trade is contributing to the expansion of existing hybrid zones. The outcomes of this thesis provide the means for the surveillance of these translocation, co-infection and/or hybridisation events in the definitive host beyond what was previously possible. By allowing ante-mortem *Fasciola* spp. differentiation, future investigations can be conducted at the source of infection and may help to elucidate information regarding the as-of-yet unknown degree of introgression of one *Fasciola* species into another. The specific areas of future research required as a result of the questions raised in this thesis are, in no particular order:

1. The comparison of faecal and post-mortem *Fasciola* spp. identification using the molecular diagnostic methods described herein. While the methods presented in this thesis are robustly validated, a direct comparison between ante- and post-mortem findings was not possible due to a lack of access to abattoir specimens (e.g. Anderson et al. (1999)). Post-treatment trials should also be conducted to confirm when animals are first considered *Fasciola*-free using molecular diagnostic methods (see George et al. (2017)). Such an investigation would resolve any outstanding questions regarding the validity of these assays and may contribute to their inclusion in molecular diagnostic panels that are currently available for the diagnosis of other economically-important parasites of livestock.

2. Investigations into the phenotypic implications of *Fasciola*-hybrids via observations of both experimental and natural infections. A direct comparison of the immune responses of definitive hosts to experimental infection with *F. hepatica, F. gigantica* and their hybrids is necessary to help answer questions regarding their pathogenicity and control (such as those previously conducted by Zhang et al. (2005) and Raadsma et al. (2007)). Continued molecular surveillance of natural infections in livestock in areas of parasite sympatry would assist our understanding of the scale and source of co-infections, and should be extended to include the diagnosis of human infections in known hybridisation hotspots. In the absence of more definitive information regarding the frequency and impacts of hybridisation and introgression between *Fasciola* spp., methods to minimise co-infection of definitive hosts, such as the commercialisation of a vaccine, should be advanced (Molina-Hernández et al., 2015).

3. The development of guidelines for the identification and treatment of *Fasciola*-positive animals prior to transport. The potential human and animal health implications of the translocation of *Fasciola* spp. as a result of the international livestock trade should be acknowledged and appropriate pre-shipping treatment and/or quarantine guidelines established. This is necessary regardless of any potential impacts of

co-infection, hybridisation and introgression, as often these animals are moved through less-developed areas where parasite control is not routinely practised and where access to medical facilities is limited.

4. The experimental back-crossing of *Fasciola*-hybrids with both parental species should be conducted to confirm the occurrence of introgression between the two species via whole genome sequencing. Genetic markers of *F. hepatica* and *F. gigantica* likely to undergo introgression should be investigated via whole genome sequencing and experimental backcrossing of *Fasciola*-hybrids with both parental species (as conducted by Redman et al. (2012) to demonstrate introgression of resistance genes in *Haemonchus contortus*). This would aid in the development of a panel of key markers, which should then be applied to adult specimens collected from areas of parasite sympatry to ultimately progress our understanding of the phenotypic outcomes of introgression between these parasites (Cwiklinski et al., 2015).

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