Identification of viable liver fluke metacercarial challenge to livestock

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Abbreviations

AHDB – Agriculture and Horticulture Development Board

DM – Dry matter

FEC – Faecal egg count

NIR – Near infrared spectrophotometry

RSBP – Royal Society for the Protection of Birds

SCOPS – Sustainable Control of Parasites in Sheep

TCBZ – Triclabendazole

VFA – Volatile fatty acids

Declaration

The work presented in this thesis is my own work, unless otherwise stated. This work has not been submitted for any other degree or professional qualification.

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Abstract

The parasitic liver fluke, *Fasciola hepatica*, presents a significant economic burden to the UK livestock industry. Liver fluke disease (fasciolosis) control is underpinned by forecasting, which is currently performed on a relatively crude regional scale, informed by traditional seasonal disease patterns and prevailing climatic conditions. The ultimate indicator of fluke infection risk is the infectious cyst (metacercarial) challenge on pasture; hence precise forecasting of the fasciolosis risk to grazing livestock requires the development of new methods to identify and quantify viable metacercariae on pasture.

The work in this thesis focuses on the development of assays to evaluate the viability of metacercariae, methods to recover metacercariae from herbage, and DNA detection techniques to identify and quantify the numbers of viable metacercariae from pasture samples. Furthermore, there is investigation and discussion of the risk of fasciolosis to livestock grazing on wetland areas as part of agri-environmental schemes, and to housed livestock consuming grass silage.

Determination of metacercarial viability is key to the prediction of metacercarial challenge on pasture. Methods which are quick and relatively easy to perform are appropriate for liver fluke forecasting as avoidance of infections is important to the livestock industry. Assessment of metacercarial morphology, using light microscopy, offers a quick and relatively easy method to determine cyst viability. Excystment assays are another method used to determine metacercarial viability in the lab, each assay varying in design and ease to perform. The reagents used and the design of ‘C. E. Bennett excystment assay’ show promise as a method which is both easy to perform and potentially applicable to the determination of metacercarial challenge from pasture on farm. The aim of Chapter 2 was to evaluate the reliability of both light microscopy and the ‘C. E. Bennett excystment assay’ as methods to determine metacercarial viability. This was demonstrated on metacercariae subjected to one well researched factor of viability, temperature, and another lesser known factor of viability, salinity.

The ability of the ‘C. E. Bennett excystment assay’ and light microscopy were evaluated to determine metacercarial viability; both were found to be unreliable. For this thesis a robust and reliable method of determining metacercarial viability was required: this was provided by the excystment method published by Hernández- González et al. (2010).

Despite the poor excystment percentages obtained using the ‘C. E. Bennett excystment assay’, it was straightforward to perform. Shop-bought carbonated water was used to modify the excystment assay published by Hernández-González et al. (2010). This modified assay gave excystment percentages of 97% using ovine bile and 90% using bovine bile with freshly encysted metacercariae. The use of carbonated water made this method more applicable to an ‘on-farm’ testing scenario.

Metacercarial recovery from pasture is essential to determine metacercarial challenge on farms/fields. The physical removal and isolation of metacercariae from grass provides information as to the number of metacercariae on the pasture. Currently, a routine method of metacercarial recovery from pasture does not exist. One aim of Chapter 3 was to demonstrate the combined use of vinegar, as a detachment solution, and a salad spinner, to isolate metacercariae from their original encystment material. This method was evaluated on *F. hepatica* metacercariae encysted on cellophane; on metacercariae allowed to re-adhere to both grass and cellophane; and on grass exposed to snails shedding metacercariae of another species of parasitic fluke, *Notocotylus*. About 44% of metacercariae encysted on cellophane were estimated to be recovered. Similarly, 44% of re-adhered metacercariae were recovered from grass, compared to 29% of metacercariae re-adhered to cellophane. The limitations of using re-adhered metacercariae to represent freshly encysted metacercariae are discussed.

In the absence of direct access to a laboratory *Galba truncatula* mud snail colony, *Radix* sp. snails shedding *Notocotylus* sp. metacercariae were placed on laboratory grown grass. Seven *Notocotylus sp.* metacercariae were recovered. The total number of *Notocotylus* sp.

metacercariae encysted upon the grass sample was unknown and because of this the efficiency of recovery could not be measured.

Another aim of Chapter 3 was to evaluate the specific risk of liver fluke to livestock grazing on wader scrapes over time. Another method of metacercarial recovery, cellophane rafts, was used to determine the risk of fasciolosis to livestock grazing around wader scrapes, established to promote biodiversity on farms. Wader scrapes can overlap with and create habitats suitable for the *G. truncatula* intermediate mud snail hosts for *F. hepatica*, causing concern to farmers who are required to graze their livestock around these areas in order to receive environmental subsidies. Two newly installed wader scrapes and a ditch located on Scottish hill farms were sampled over three years. Cellophane rafts were deployed in all three areas in an attempt to collect any available metacercariae. Snail numbers were monitored and specimens were collected. Faeces from both wildlife and livestock were collected opportunistically and fluke egg counts were performed. A total of 198 snails, either *G. truncatula* or *Radix sp.* were collected over the 3 years. 2.5% of these snails were positive for *F. hepatica* as determined by PCR. 10% of sheep faecal samples collected were positive for *F. hepatica,* as determined by faecal egg count. Of the 7 deer samples collected during the three year study period, one had a *F. hepatica* egg count of 1.67 eggs per gram. Putative metacercariae were observed on the cellophane rafts, but their identity could not be determined. It was concluded that wader scrapes could offer suitable habitats for *G. truncatula* and that there is a potential risk of fasciolosis to livestock grazing on these areas. It is important that farmers appreciate the risk relative to where livestock are grazing and the health status of their stock.

The risk of fasciolosis to housed livestock fed grass silage is unknown. Silage fermentation is a many factorial process, Tarczynski & Podkowka (1964) suggested that pH impacts the survival of metacercariae when ensiled. Chapter 4 aimed to determine the longevity of viable *F. hepatica* metacercariae at different pH associated with silage fermentation, and to determine the longevity of *F. hepatica* metacercariae in silages of different qualities, produced under laboratory conditions.

The modified Hernández-González et al. (2010) excystment method was used to determine metacercarial viability after incubation at different pH associated with silage fermentation. Metacercariae were incubated for 4 weeks in lactic acid solutions of pH 4, 5 and 6: viability remained high, being 88%, 82% and 91%, respectively. A longer exposure to lactic acid, pH 4, resulted in relatively high viability over a period of 12 weeks, but after 16 weeks, the percentage viability was significantly lower ($p=0.008$) than in the comparative control group; 38% vs. 72%, respectively.

Small aliquots of pre-made grass silage were used to replicate ensiling conditions in the lab and to test metacercarial viability. Silages of 'high', 'medium' and 'low' quality were sampled from farms in Wales. These samples were split and spiked with *F. hepatica* metacercariae. Time intervals of 4, 8, 12 and 16 weeks were employed for the recovery of metacercariae from the 'high' quality silage sample. Recovery of metacercariae from the 'medium' and 'low' quality silages was determined at one time point of 14 weeks after spiking with fluke cysts. Metacercarial recovery was inconsistent regardless of time point or silage quality. Viability was determined using the modified Hernández- González et al. (2010) excystment method. The only group to contain viable metacercariae was the 'high' quality silage after 4 weeks of incubation. 16% of the metacercariae present in this sample of 'high' quality silage were recovered; 58% of the recovered metacercariae were viable. The multifactorial nature of grass silage fermentation means that fully understanding metacercarial survival in silage is not straightforward. pH alone does not appear to be the main determinant of cyst viability. Metacercariae could survive well in pH 4 lactic acid solution, but did not survive well in equivalent pH silage. It was concluded that it is possible for metacercariae to survive in silage, but the risk of fasciolosis when well-made silage is fed to livestock is very low.

DNA methods are used routinely in applied research to identify *F. hepatica* in snail DNA and faecal DNA samples. These methods are highly specific and highly sensitive, highlighting the benefit that they could have for the detection of *F. hepatica* metacercariae from pasture samples. Polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) were the methods selected for the development of DNA-based detection of *F. hepatica* metacercariae from pasture samples. The work of Chapter 5 aimed to demonstrate the use of these methods and their effectiveness using DNA extracted from different prepared DNA samples. The mitochondrial cytochrome c oxidase subunit 1 (COX1) target was amplified from both DNA extracted from grass spiked with metacercarial DNA, and from grass spiked with viable metacercariae. The sensitivity levels of each assay were determined using DNA dilution series in water and in DNA extracted from grass. LAMP had a 100 times greater sensitivity than the equivalent PCR (0.001ng/µl vs 0.1ng/µl) when metacercarial DNA was diluted in grass DNA. LAMP was able to detect the target at a concentration of 0.001 ng/µl in 30 minutes. PCR and LAMP both showed capability of detecting metacercariae from grass samples. However, the translation of these tests for use with farm samples may not yet be practically feasible. DNA testing does not give any indication of the source of DNA being amplified: it cannot be known if the DNA amplified from a pasture sample has originated from a *F. hepatica* eggs, miracidia, cercariae, or metacercariae. Similarly, the results of a DNA test performed on pasture samples would be of little help to farmers without knowledge of the viability status of metacercariae amplified.

The main findings of this thesis are: (1) metacercarial viability cannot be reliably determined using light microscopy or the ‘C. E. Bennett excystment assay’; (2) the modified Hernández-González et al. (2010) method is an efficient excystment method; (3) in the absence of a mud snail colony, demonstrating methods of metacercarial recovery and isolation is challenging; (4) there is a potential risk of fasciolosis to animals grazing on wader scrapes; (5) *F. hepatica* metacercariae are able to survive in grass silage for a short time; and (6)

LAMP is x100 more sensitive than PCR and it is the most applicable method for DNA detection of metacercariae from grass.

Overall, this work highlights that with future development of cyst recovery and viability techniques, the metacercarial challenge on pasture could be effectively quantified on farms. This would allow for more precise and accurate determination of liver fluke risk in time and space.

Lay summary

The parasitic liver fluke, *Fasciola hepatica*, presents a significant economic burden on the UK livestock industry. An estimated £300 million per annum has been attributed to the impact of fasciolosis in UK sheep and cattle. The emergence of drug resistant fluke populations and increasing reports of 'unseasonal' fluke disease associated with over-winter survival of fluke stages on pasture have been linked to higher prevalence of *F. hepatica* infection in recent years. Fluke infections occur when animals ingest infective, dormant cysts (metacercariae) on pasture while grazing. Faecal and blood diagnostic tests only detect disease after the animal has become infected. Currently, forecasting liver fluke risk to livestock is performed at a relatively crude, regional scale, based on traditional seasonal disease patterns and prevailing climatic conditions. However, farmers are concerned about fluke risk at a more local level, specific to their farm and fields, for which no detection method is currently available. The ultimate indicator of fluke infection risk is the metacercarial cyst challenge on pasture. The overall aim of this project was to develop methods for the detection and identification of fluke infective stages on pasture samples.

The work in this thesis begins with the evaluation of methods to assess metacercarial viability. Visual assessment of metacercariae, under a microscope, can indicate if metacercariae are alive or dead, while excystment assays mimic the process of the juvenile fluke emerging from the cyst wall within the gut of the host species. Development of techniques to determine metacercarial viability is important for understanding and predicting liver fluke risk on pasture. The number of live metacercariae on pasture will inform as to whether the liver fluke risk is high or low for a farm/field.

Chapter 2 demonstrates the reliability of microscopy and the 'C. E. Bennett excystment assay' to determine metacercarial viability. These methods were chosen because of their simplicity to perform and their potential to be used as 'on farm' tests. Both microscopy and the 'C. E. Bennett excystment assay' were shown to be unreliable methods of determining

metacercarial viability, while the excystment method published by Hernández- González et al. (2010) was highly efficient. The application of this viability assay as an ‘on farm’ test shows promise, and was used in this thesis to test metacercarial viability under different conditions.

The physical retrieval of metacercariae from pasture is valuable to the understanding of how many metacercariae are present on pasture at any one time. Collecting metacercariae from pasture can also be advantageous for the many tests that can be performed on them. Collected metacercariae can be tested for their viability, as previously described, and their species identity can be confirmed by DNA techniques. Chapter 3 demonstrates the recovery of metacercariae from grass and cellophane. Vinegar was used to detach metacercariae from each substrate and a salad spinner was used to separate the substrate from metacercariae. The recovery of metacercariae from grass and cellophane was challenging but future development of these methods could provide an effective means of quantifying metacercarial numbers on pasture.

Wader scrapes are habitats purposefully created on farms to provide an environment for wetland birds and insects. They also have the potential to provide habitat for the intermediate host of liver fluke, *Galba truncatula.* Under some environmental stewardship schemes, there is a requirement for these areas to be grazed with livestock. This concerns some farmers about the potential risk of liver fluke infections in their stock. The risk of fasciolosis to livestock grazing wetland areas, such as wader scrapes, was investigated in Chapter 3. As a way of collecting metacercariae, cellophane rafts were deployed in newly established wader scrapes and a ditch of high risk field on the same farm. Snails and faeces were also collected from these areas to survey the risk of liver fluke over time. It was concluded that livestock grazing on wader scrapes have a small chance of becoming infected with *F. hepatica*, but the risk must be evaluated by farmers in relation to the reality of the situation on their farm.

The risk of liver fluke infection to livestock fed on grass silage is unknown. Farmers have expressed concern that grass silage is the source of infection in housed livestock who have limited access to the outdoors. Silage pH has previously been identified as a possible factor in the survival of metacercariae in grass silage. pH can also indicate the quality of silage. The survival of liver fluke metacercariae in different pHs associated with silage fermentation was evaluated in Chapter 4. The survival of metacercariae in small grass silage bags of different qualities was evaluated. It was found that metacercariae can survive for a short time in silage, and that pH is not the determining factor of metacercarial survival. The risk of liver fluke infection to livestock is still unknown because silage fermentation is variable and many factors are involved in this process. However, provided that farmers manage areas of potential snail habitats in their grass fields and carry out responsible control practice on their farms.

DNA testing methods offer powerful tools for the determination of liver fluke risk of pasture. These methods have advantages over the slow process of identifying high risk areas on fields by snail surveys or by the recovery of metacercariae from pasture samples. In Chapter 5, DNA methods for the detection of metacercariae from pasture samples were demonstrated. Two methods of DNA amplification were evaluated. Polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) were compared in their performance to amplify metacercarial DNA from grass. LAMP was able to detect metacercarial DNA concentrations x100 smaller than the limit detected by PCR in the presence of grass extractants. The results of a DNA assay performed on a pasture sample from a farm would confirm *F. hepatica* presence but would not inform farmers of the viable metacercarial risk. The high specificity of DNA testing would allow for accurate determination of species. Realistic application of DNA testing for pasture samples would be done as an additional test, after metacercarial recovery and viability assays.

The methods developed in this thesis have potential to give farmers information as to the quantity, viability and species identity of metacercariae on their pasture. The combined power of this knowledge will improve our understanding of fluke risk on farms and help identify disease risk with adequate time to inform avoidance strategies and facilitate targeted disease control.

Chapter 1: Introduction

1.1 Liver fluke biology

Fasciola hepatica, commonly known as the liver fluke, is a leaf-shaped parasitic flatworm (trematode) capable of infecting a wide range of mammalian hosts (Dalton, 1999). In the United Kingdom (UK), *F. hepatica* is responsible for most cases of fasciolosis, with significant infections occurring in sheep and cattle.

This parasite, like many other trematodes, has a complicated, indirect lifecycle (Figure 1.1). Adult fluke residing in the bile ducts of their host lay eggs which are excreted onto the pasture in the host faeces. Given suitable climatic and environmental conditions, microscopic ciliated larvae (miracidia) hatch from the eggs and go on to locate and penetrate the intermediate host, the mud snail, *Galba truncatula.* There are several replicative stages within the mud snail; miracidia turn into sporocysts, sporocysts generate rediae and rediae produce cercariae (Andrews, 1999). The larvae within *G. truncatula* asexually multiply, a single sporocyst can give rise to many cloned rediae and cercariae (Dreyfuss, Vignoles & Rondelaud, 2015).

Environmental conditions – most significantly, rainfall and temperature – dictate both the rate of development of cercariae and the success of cercarial shedding, which can range between 5-12 weeks post-infection (Dreyfuss, Vignoles, & Rondelaud, 2015). Once free of the snail body, the cercariae use their tail to find a suitable plant on which to encyst as infective metacercariae. It can take between a few minutes and 2 hours for the free-swimming cercariae to complete the journey from snail to place of encystment (Andrews, 1999). Definitive hosts will ingest metacercariae as they graze infested pasture or herbage. In response to physico-chemical conditions, namely increased carbon dioxide, reducing conditions and temperature of around 39 °C, metacercariae are activated in the rumen or stomach of the host (Dixon, 1966). The presence of bile causes the second stage of excystment which is the emergence of the juvenile fluke within the small intestine (Dixon, 1966). The newly excysted fluke then make their way through the intestinal wall and into the body cavity where they migrate towards the liver. The juvenile fluke spend the first 5-6 weeks of infection burrowing through the liver till they reach the bile ducts, as adults. The fluke mature in the bile ducts and begin to produce eggs (up to 25,000 per day) which are eventually passed out in the faeces of the host in to the environment (Behm & Sangster, 1999).

The severity of disease (fasciolosis) experienced by the host is dependent upon many factors including the hosts current nutritional and health status and the number of viable metacercariae ingested over a period of time. Most commonly, host animals, such as sheep, will become infected with a constant small number of metacercariae over the course of the grazing season, leading to a prolonged steady build-up of infection (Behm & Sangster, 1999). Where the climatic conditions favour *F. hepatica* and the grazing management of stock allows, many metacercariae can be ingested at one time (Behm & Sangster, 1999). The simultaneous excystment and migration of juvenile fluke through the body, especially the liver tissue, can have serious health implications and even lead to sudden death of the host.

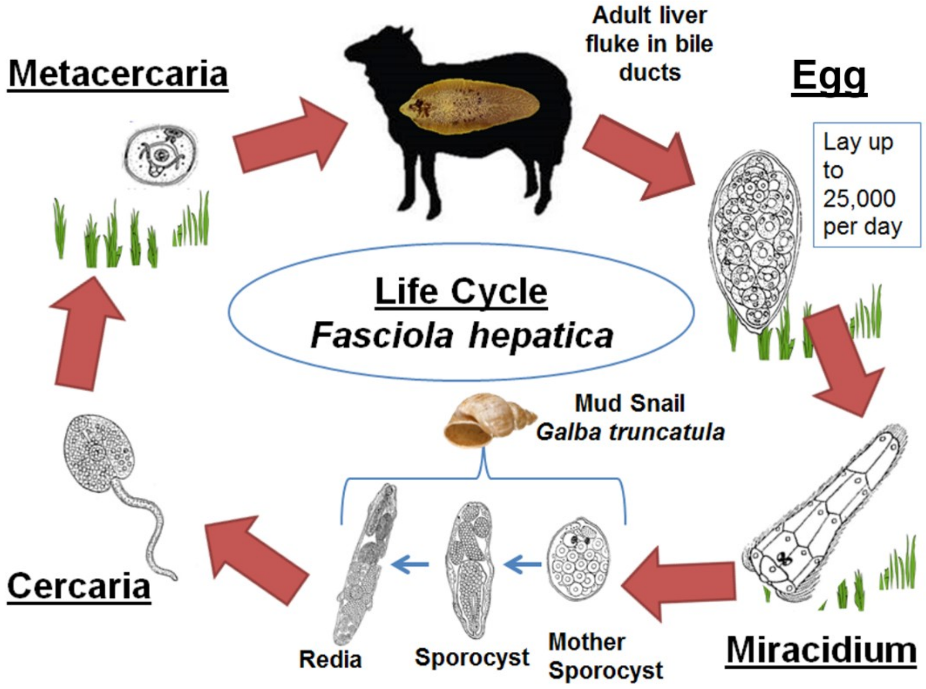

Figure 1.1 – Lifecycle of liver fluke (*Fasciola hepatica*)

1.2 Distribution

The global distribution of *F. hepatica* covers the temperate regions of the continents of Europe, North and South America, Africa, Asia and Australia (Mas-Coma, Bargues, & Valero, 2005). *Fasciola gigantica,* a close relative of *F. hepatica,* is found in tropical climates, with confirmation of presence in Africa, Eastern and Southern Asia and Eastern Europe (Torgerson & Claxton, 1999). In the UK, cases of fasciolosis were traditionally associated with the wetter western areas of the island, particularly in Wales and Scotland (Mitchell, 2002). These areas offered favourable climatic conditions, namely high rainfall and mild temperatures, suitable for the habitat of the intermediate mud snail host and free-living stages.

The liver fluke life cycle, in the UK, traditionally begins in May with the hatching of eggs and subsequent infection of snails, which are also multiplying during this time (Taylor, 2009). Moisture and temperatures above 10°C are required for the hatching of *F. hepatica* eggs and the activity of *G. truncatula*. In the UK these conditions occur between the months of May and October (Taylor, 2009). From September, cercariae are shed by the snails and encyst on to pasture as metacercariae. This step is also dependent on moisture and temperature, with plenty of moisture and warm conditions allowing for the survival and activity of snails and for the shedding of many cercariae. Inversely, if conditions are dry and cold during the summer then snail numbers suffer and consequently the numbers of metacercariae on pasture is lower (Taylor, 2009). The standard description of *G. truncatula* habitat is muddy, juncaceous, wet areas subject to periods of intermittent flooding (Dreyfuss, Vignoles & Rondelaud, 2015). Colloquial terms used by farmers to describe areas of snail habitat and or high fluke risk on fields include, ‘boggy, ‘rushy’ and ‘fluky’.

An increase in the incidence of fasciolosis has been identified in recent years. The season of 2012/13 was a particularly bad year, with record number of cases attributed to a very wet summer, a mild winter and extensive flooding (Skuce, van Dijk, Smith, & Morgan, 2014). High risk years such as these are most likely the product of climate change and there is evidence from the past 30 years to suggest that this has led to changes in the epidemiology of parasites such as *F. hepatica* (van Dijk, Sargison, Kenyon, & Skuce, 2010). The threat of fasciolosis in the UK is predicted to increase in most parts of the country with western areas incrementally experiencing increasing widespread fasciolosis from 2020 to 2070 (Fox et al., 2011).

1.3 Clinical signs

Infections of *F. hepatica* can lead to disease; fasciolosis. The destructive movement and blood-feeding habit of newly-excysted juvenile flukes and/or adult liver fluke causes harm to the definitive hosts. The basis of this project focuses on the development methods to help forecast disease risk for sheep and cattle. Other hosts include; humans, deer, goats, and pigs, but disease in these species is rare and methods developed for this project are not applicable to these types of cases.

Animal losses will begin 2 months after their introduction to high risk pasture (Behm & Sangster, 1999). The severity of the infection is partly determined by the number of viable metacercariae ingested and the time course involved. The general health status of potential hosts will also determine the severity of the infection, with different cases of severity presenting in a single flock or herd of livestock (Behm & Sangster, 1999). Age, weight, nutritional status, the other environmental stressors can influence the effect of the infection on animals (Burden et al., 1978).

Sheep

Ovine fasciolosis can present in three different forms: acute, subacute and chronic. A host animal will suffer from acute fasciolosis after the ingestion of many metacercariae over a short period of time (6-8 weeks) (Behm & Sangster, 1999). Migration of juvenile fluke through the gut wall, after excystment, and their journey through the body towards the liver parenchyma causes considerable damage to the host. On their passage, fluke will dissolve the gut wall and the host can suffer major haemorrhage if many fluke migrate at once. Clinical signs include sudden death (in cases of >5000 metacercariae ingested), abdominal haemorrhage, and recumbency (Behm & Sangster, 1999).

Sub-acute fasciolosis is experienced in hosts 12-40 weeks after infection and is associated with the recovery of >800 flukes. Lethargy, anaemia and weight loss are the clinical signs presented in sheep (Behm & Sangster, 1999). Although many cysts may have been ingested by the animal, clinical signs present later because the number of cysts ingested during that time has not been great enough to establish acute fasciolosis (Mitchell, 2002).

Chronic disease develops gradually, the accumulation of >200 flukes in the liver resulting from the ingestion of a small number of metacercariae over time (Behm & Sangster, 1999). The livers of chronically diseased sheep are paler than their usual colour, puckered with old fluke tracks and the bile ducts are visibility bigger due to the walls of the ducts thickening. Bottle jaw, emaciation and anaemia are the associated clinical sings of chronic fasciolosis (Behm & Sangster, 1999).

Cattle

Cattle are less likely to suffer from acute fasciolosis than sheep; the lack of cases is attributed to the size and resilience of cattle livers which are tough and fibrous (Mitchell, 2002 and Skuce and Zadoks, 2013). Sheep have smaller livers and typically the smaller the liver the greater the overall impact of a liver fluke infection on the organ and its ability to function normally (Behm and Sangster, 1999). Cattle can also withstand higher burdens of fluke; calves showed no signs of clinical disease when infected daily with 20 *F. hepatica* metacercariae for 20 weeks (Burden et al., 1978). A mean of 200 adult fluke were recovered from each calf and each calf gained an average of 990g per day. It should be noted that these calves were housed and fed high quality feed (Burden et al., 1978). Conversely, sheep infected with >200 fluke suffered greater weight loss and emaciation when fed a low protein diet compared to their high protein fed, also infected, counterparts (Berry and Dargie, 1976).

Sub-clinical disease is more common in cattle and the economic losses occurred through blood loss and damage to the liver are often attributed to other causes (Mazeri, et al., 2017). Underdiagnosis of fasciolosis in cattle maintains a cycle of pasture reinfection, reduced productivity and chronic disease in the animals.

Cases of acute and sub-acute fasciolosis are usually observed during late autumn and early winter, especially after a wet summer. Cases of chronic fasciolosis are usually observed in late winter/early spring and are associated with prolonged infection, originating from a persistent low level of exposure to metacercariae. Over time, the numbers of adult fluke build up in the bile ducts of the host where they feed on hepatocytes and blood. Lesions and calcification of the bile ducts, a condition known as pipestem fibrosis, is commonly associated with chronic infestations in cattle and liver condemnation caused by fasciolosis (Figure 1.2) (Gordon-Gibbs, 2014). The destructive feeding behaviour of the adult flukes is painful for the animal and they will consequently suffer from weight loss, poor body condition and anaemia (Behm and Sangster, 1999).

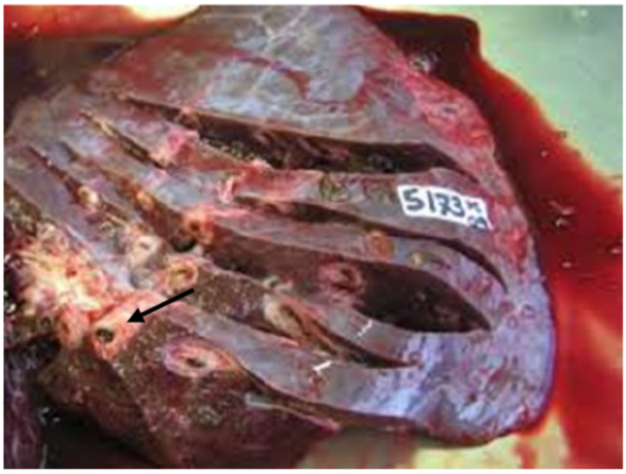

Figure 1.2 Bovine liver biliary hyperplasia from chronic fluke infection (Toolan, 2009). Note the thickened bile ducts (pipestem fibrosis), see arrow.

In business terms, farms can suffer economically, losing money through the overall effect of fasciolosis on their livestock and through the costs of control and treatment associated with this disease. The economic significance for farmers and the UK livestock industry is explained more below.

1.4 Economic significance

Globally, the cost of liver fluke has been estimated to be €2.5 billion (European Commission, 2012). The high cost of fasciolosis is exacerbated further when considering the current state of the economy and tight profit margins for farmers in the livestock sector. The UK livestock industry incurs significant losses from the effects of fasciolosis in livestock. Examples of this include death of animals, reduced productivity, treatment costs, reduced feed conversion ratios (W. Thomson, Harbro Ltd., pers comm with P. Skuce), higher feed costs (Sargison and Scott, 2011), poor reproduction (Schweizer, et al., 2005) and reduced profit due to condemned livers at the abattoir. During 2012 in England, it was estimated that approximately 16% of cattle livers (259,000) and 7% (582,000) of sheep livers were condemned at slaughter (EBLEX, 2013). In total, £3 million was attributed to condemned livers because of damage caused by liver fluke (EBLEX, 2013). In 2013, Harbro Ltd published data from a comprehensive 3-year study on the effect of liver fluke. This included information from ~450,000 beef cattle sourced from North East Scotland. It was found that, on average, those animals infected with liver fluke, as determined by meat hygiene inspectors, were 27 days older and 2.5kgs lighter than their 'fluke-free' counterparts at slaughter. This amounts to a direct cost of £60 per head dead weight (W. Thomson, Harbro Ltd., pers comm with P. Skuce). In 2016, a NADIS bulletin estimated that the total yearly cost of fasciolosis to the UK cattle industry was £23 million (NADIS, 2016). This figure probably does not reflect the actual cost of fasciolosis to the UK cattle industry but rather underestimates the cost. Figures attributing to the cost of fasciolosis do not include losses from undiagnosed cases or subclinical disease. It is difficult to accurately determine the actual cost of fasciolosis to the UK cattle industry because of the difficulty in understanding the complex relationship between fluke burdens and production loss, which is influenced by many different factors (Mazeri, et al., 2017). The level of challenge on the pasture, how long livestock graze contaminated pasture, and the animals metabolic and immune response to the parasite are a few of the factors involved (Sykes, 1994).

Despite the challenge of determining production losses attributed to fasciolosis, the examples cited highlight the serious cost of this disease and the damaging effect this could have on farm businesses. Reducing physical losses caused by fasciolosis would be economically beneficial. Economically, the industry could improve with reduced cases of fasciolosis as there would be more stock available for sale, milk; and wool production would increase, and there would be less money spent on treatments, although this is not where the majority of losses occur. In an estimation of the losses incurred due to fasciolosis, milk yield was the worst affected with a median annual loss of €33,847000 (Schweizer, et al., 2005). Treating milking cattle for fluke is challenging because there isn’t a suitable flukicidal drug to administer that has a reasonable withdrawal period and can kill the youngest stage of fluke (Schweizer, et al., 2005). Grass fed dairy herds can use tactical grazing patterns and pasture management as a means of fluke control but in high risk areas this can be difficult.

There exist many complicated risk factors which hinder responsible control of fasciolosis and large-scale control of liver fluke, particularly, the rise in incidence of flukicidal resistance, climate change, wildlife and agri-environment schemes for increasing biodiversity on farms. Therefore, farmers must now place a greater reliance upon preventative measures.

1.5 Risk Factors

1.5.1 Anthelmintic resistance

Flukicides are part of a group of drugs called anthelmintics, which are employed by vets and farmers to control a number of gastrointestinal parasites. Fluke-infected animals are dosed with flukicides to both prevent clinical disease and reduce recontamination of pasture. Unlike some wormers, flukicides have no residual effect; these drugs only work on the day of treatment and don't provide protection over subsequent weeks (Skuce and Zadoks, 2013).

The most commonly used flukicide, triclabendazole (TCBZ), kills the widest range of fluke stages within the definitive host (from 2 weeks old fluke and onwards) (Table1) (Fairweather and Boray, 1999). It is the only drug that can effectively target the damaging early immature flukes in livestock (Boray et al., 1983). This has made TCBZ a very popular flukicide with farmers because, unlike other flukicides, there is no need to pay for diagnostic tests in order to determine the age of the fluke present so the correct flukicide can be administered (Kelley et al., 2016). TCBZ is used commonly to protect sheep from acute fasciolosis and reduce losses in flocks as they graze high risk pasture in the autumn. For many farms, sheep grazing in autumn is only possible because of the use of TCBZ and its use has become an important part of fluke control on these farms (Patrick, et al., 2018).

Resistance to TCBZ has now been confirmed in the UK (Gordon, Zadoks, Skuce, & Sargison, 2012; Sargison, 2012 and Hanna et al., 2015), Europe (Moll et al., 2000 and Alvarez-Sanchez et al., et al 2006), Australia (Overend & Bowen, 1995) and South America (Olaechea et al, 2011; Ortiz et al., 2013). Development of resistance to the most effective class of drugs to treat fasciolosis has increased the threat of liver fluke to livestock health, welfare and productivity (Howell et al., 2015). An example of TCBZ treatment failure on one farm in South East Scotland was calculated to cost a total of £19,200 (£8.73 per ewe) in one grazing season (Sargison and Scott, 2011).

It has been suggested that resistance to TCBZ was assisted by a lack of education to farms on the correct dosing procedure, the correct drugs to use, the importance of testing for drug efficacy and more generally on the biology of liver fluke (Kelley et al., 2016). TCBZ resistance in *F. hepatica* has also been shown to spread genetically by the clonal expansion of the life stages within *G. truncatula* (Hodgkinson et al*.,* 2018).

Diagnosis of TCBZ failure is difficult. Many of the factors that have been identified as contributing such as incorrect dosing through faulty equipment, incorrect technique, poor drug storage and dosage calculations can also lead to cases of mistaken TCBZ resistance (Fairweather, 2011). Determining the difference between treatment failure and parasite resistance is difficult, especially in the absence of an approved and standardised faecal egg count reduction test for trematodes (FECRT) (Coles et al., 2006). With the rise in presumed cases of TCBZ failure, farmers could be resorting to unnecessary and incorrect drugs for treatment at inappropriate times, thus making the current situation worse (Fairweather, 2011). A systematic approach, including the consideration of other causes, must be taken when examining cases of apparent TCBZ treatment failure. Underdosing, re-infection, expired products and improper storage of product are all potential causes of treatment failure (Patrick et al., 2018).

Albendazole, oxyclozanide, nitroxynil and closantel are four other active ingredients used in flukicides. Their individual percentage efficiency in sheep and the age of fluke each drug targets is described in Table 1.1 (Fairweather and Boray, 1999).

Table 1.1 – Anthelmintic percentage efficiency, modified from Fairweather and Boray (1999)

Drug	Age of fluke (weeks)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Albendazole										50-70%		80-90%		
Oxyclozanide										50-70%		80-90%		
Nitroxynil							50-90%			91-99%				
Closantel							50-90%			91-99%				
Triclabendazole		90-99%		99-100%										

Albendazole is commonly prescribed to treat against fluke but it is only effective in adult fluke of 10 weeks and older (Table 1.1). Fairweather and Boray (1999) report efficiency of up to 90% in fluke over 12 weeks but Coles and Stafford (2001) report higher efficacy, 94-95%. There are reports of albendazole resistant populations of fluke in Spain (Alvarez-Sánchez et al., 2006) and Sweden (Novobilský et al., 2012). No reports of albendazole resistant fluke populations in UK (Patrick et al., 2018). Nitroxynil and Closantel are effective against fluke aged 7 weeks and older (Fairweather and Boray, 1999). Closantel was also described to have 23-73% efficiency against fluke aged 3-4 weeks, 91% for 5 week old fluke, 91-95% for 6-9 week old fluke and 97-100% for 10-14 week old fluke (Williams, 2020). A case of closantel resistance in fluke from cattle has been reported in Sweden (Novobilský and Höglund, 2015). Development of resistance to the flukicides albendazole and closantel in fluke populations is concerning when considering that these drugs can be used to control TCBZ resistant populations (Coles and Stafford, 2001).

Reliance solely on the use of anthelmintics against liver fluke is not a sustainable method of control for farmers (Sargison, 2014). Although flukicides are still an important tool in the control of liver fluke, farmers are also encouraged to employ alternative strategies to reduce their reliance on flukicides. Fencing off boggy areas and drainage of land that could act as the habitat for *G. truncatula* is recommended to reduce the potential for grazing land to harbour infective liver fluke metacercariae (Sargison and Scott, 2011). Farmers are encouraged by industry groups such as SCOPS (Sustainable Control of Parasites in Sheep) to adopt this method of fluke management rather than solely relying on the regular treatment of stock with flukicides. There are limitations to this strategy for some farms where most of the land would be deemed high risk, but this method of control is considered to be important but underused by farmers (Skuce and Zadoks, 2013).

The planting of trees in high risk fluke areas in order to reduce *G. truncatula* was discussed by Richards (2016). The soils of woodlands have a lower moisture level than grasslands and are unsuitable areas for *G. truncatula* habitat. The strategic planting of trees in high risk areas of grassland would reduce populations of *G. truncatula* and would also make the same areas unsuitable for liver fluke stages on the pasture, thus reducing the risk of liver fluke to livestock (Richards, 2016).

G. truncatula have been observed in the muddy areas around water troughs (Knubben-Schweizer and Torgerson, 2015). Where possible water should be provided to stock in non-leaky troughs or in troughs installed on concrete as this removes muddy areas which could provide snails with micro-habitats (Patrick et al., 2018). Other strategic measures for managing snail populations on farms include the rotation of fields with crop and grazing. Egg shedding can be managed by treating housed stock prior to grazing (Patrick et al., 2018)

With the encouragement of farmers to reduce reliance of flukicides and adopt more sustainable fluke control measures, the use of flukicides will still remain a key tool for the control of fasciolosis. The development of a vaccine against *F. hepatica* is considered to be the most important research topic and despite the best efforts of many experienced research groups, a vaccine for the control of fasciolosis is currently not available (Molina-Hernandez et al., 2015). Issues facing the development of a successful vaccine include lack of understanding of how *F. hepatica* evades the host immune response, the correct antigens and delivery to stimulate protective immunity, the genetic variation of fluke in the environment, and development of a vaccine to make it commercially viable (Molina-Hernandez et al., 2015). Potential vaccine candidates have been shown to reduce fluke burdens by 43% and the egg output by 99% (Turner et al., 2016). The same publication also estimated that for a vaccine to be effective it must offer protection to 90% of dosed animals (Turner et al., 2016). There is very little chance of liver fluke eradication but with a vaccine liver fluke infections and fasciolosis can be reduced.

1.5.2 Climate change

Fasciolosis is historically a seasonal disease, with the acute form predominantly diagnosed in autumn and chronic infections in winter and early spring. Traditionally, fasciolosis was associated with wetter western parts of the UK. The geographical range of fasciolosis in Scotland has expanded from the ‘wetter’ farms of the west coast to drier eastern farms, where this parasite had rarely been encountered before (Kenyon et al., 2009). In recent years, climate change has created shorter, milder winters and wetter summers in the UK (Kenyon et al., 2009). Increased rainfall, coupled with warmer temperatures, has led to expansion of habitat suitable for *G. truncatula*. As a result, the survival of *G. truncatula* and life stages of liver fluke on the pasture has also increased (Mitchell, 2002; Howell, et al., 2015). This has led to an overall increase in the risk of fasciolosis across the UK and reports of fasciolosis earlier in the year from the survival of free-living stages on pasture during the winter (Fox et al., 2011).

Fasciolosis is now considered a year-round problem; the survival of *F. hepatica* eggs, infected *G. truncatula* and metacercariae on pasture over the winter allows outbreaks to continue into the spring and summer (Mitchell, 2002). The winter of 2012/2013 had the highest incidence of liver fluke in recent years and was attributed to the relatively wet and warm conditions over the previous year (Skuce & Zadoks, 2013). Events such as this highlight rapid epidemiological changes in *F. hepatica* and show, the risk of more unforeseen outbreaks occurring in the future (van Dijk, Sargison, Kenyon, & Skuce, 2010).

Extreme weather events such as flooding - although possibly beneficial in the first instance for *F. hepatica* survival - could impede the lifecycle. High rainfall could prevent the infection of snails and the encystment of metacercariae if miracidia, snails and cercariae are washed into previously fluke-free areas (Fox et al., 2011). Although the high temperatures and low moisture associated with droughts are not favourable for the survival of *F. hepatica* there is evidence that drought years lead to years of unprecedented levels of fasciolosis (Fox et al., 2011). It was suggested that during drought years the poor growth of low risk pasture forces sheep and cattle to graze rougher pasture, coinciding with *G. truncatula* habitat and higher fluke risk (Ollerenshaw, 1966).

F. hepatica has an intimate relationship with the environment. The requirement of an intermediate mud snail host has largely influenced this. Prevention of fasciolosis on farms has traditionally involved the removal of suitable mud snail habitat, but now there are government initiatives which encourage farmers to retain or introduce wet areas in their land which maybe suitable for the *G. truncatula* and potentially be high risk areas for fluke.

1.5.3 Agri-environmental schemes (AECS)

Under environmental stewardship schemes, farmers are encouraged to maintain and create wetland habitats for wetland birds and invertebrates (Scottish Government, 2016). These areas have been suggested to act as possible sources of fluke and that their creation establishes permanent high-risk areas on farms as they could overlap with the habitat for *G. truncatula.* The presence of *G. truncatula* in these areas is not known and can only be estimated at this point through knowledge of known habitats. There is, understandably, concern amongst farmers about the risk of fasciolosis because of the potential overlap with *G. truncatula* habitat, but there is little to no evidence of fluke risk in these areas. It is proposed that an established colony of *G. truncatula* could become infected with *F. hepatica* through transmission from wildlife reservoirs such as deer, rabbits and hares. Red deer are known carriers of *F. hepatica* in the Scottish highlands, with prevalence ranging from 9.6 to

53% (French et al., 2016). The risk of fasciolosis in flocks and herds is heightened further by the legislative requirement of farmers to graze animals on these areas in order to create feeding habits for birds like the lapwing and snipe (Scottish Government, 2017 and RSPB, 2017). In conjunction with the preservation of specific habitats, the dispersal of aquatic organisms, including *G. truncatula*, by wetland birds has been implicated in the spread of this gastropod around the world. It has been suggested that by incomplete digestion or by attachment to the body of birds, *G. truncatula* has been dispersed to new areas (van Leeuwen, 2012). This could widen the distribution of *F. hepatica* to areas not known to be high risk, or to newly-established wader scrapes under government schemes.

Contrary to this, farmers are advised to drain and remove areas suitable as habitat for *G. truncatula* in an effort to sustainably control fluke. Farmers are also advised, by organisations such as SCOPS, not to graze animals on potentially high-risk areas at high-risk times. Consequently, there remains a direct contradiction between the codes of practice referring to the control of liver fluke for livestock and policies aimed at the conservation of wetland habitats to promote biodiversity. To attain a balance between these two competing objectives, there will need to be improved engagement between environmental policy teams, land managers and livestock farmers.

One example of this is conservation grazing. The natterjack toad, *Epidalea calamita*, is Scotland's rarest amphibian, found only along the Solway coast in SW Scotland. They can be found in saltmarshes or 'merse' where they lay their spawn and utilise the surrounding coastal farmland to forage and hibernate (Minting & Seymour, 2016). Similar to wading birds, the natterjack toad requires short-grazed grass in order to feed but there are reports from organisations, namely, The Amphibian and Reptile Conservation Trust (ARC-Trust) and Scottish National Heritage (SNH) of unwillingness from farmers to graze these areas, due to a perceived, but unknown, risk of liver fluke infection to their livestock.

G. truncatula is seldom reported in tidal areas and is only found in waters of less than 5% salinity (Styczyncka-Jurewicz, 1966). Additionally, salinity levels compromise the development of *F. hepatica* eggs with 16% salinity being the upper limit for the hatching of miracidia (Styczyncka-Jurewicz, 1966). No data exist reporting the effect of salinity on metacercarial viability, however, the previous information may indicate that saltmarsh areas offer little risk of liver fluke to grazing livestock, and that grazing saltmarshes might offer a potential method of liver fluke avoidance (Minting and Seymour, 2016). Grazing saltmarsh areas with sheep and cattle will promote the habitat of the natterjack toad, aiding in its conversation whilst also interpreting the liver fluke cycle and potentially protecting grazing livestock from liver fluke infection.

1.5.4 Risk of fasciolosis from silage

The survival of liver fluke metacercariae in grass silage fermented in bales or pits has been highlighted by farmers as a possible disease risk to livestock that are fed silage. The same farmers also stressed that fasciolosis was occurring in livestock that had been housed, fed silage and had not been grazed outside. The Agricultural and Horticulture Development Board (AHDB), funders of this PhD project, have expressed interest in the results of an investigation of the effect of pH on *F. hepatica* metacercariae. Tarczynski and Podkowka (1964) tried to determine the viability of *F. hepatica* metacercariae on grass fermented in jars. It was reported that metacercariae lost their ability to infect rabbits within the first few days of fermentation, suggesting that death of the cysts was caused by a dramatic decrease in pH due to the creation of lactic acid (Tarczynski and Podkowka, 1964).

1.6 Diagnostic tools and control strategies

Currently there are several diagnostic tools available to determine current or historic infections of *F. hepatica* in the live animal. Some tests are more suitable than others, Gordon-Gibbs (2014) evaluated and discussed the effectiveness of each test.

The faecal egg count (FEC) is the traditional method of identifying fluke eggs from faeces. Samples of faeces can easily be collected and processed using either sedimentation or flotation techniques to determine the number of eggs per gram of faeces. Results are obtained quickly and cheaply and this may explain why this is still the mostly widely used diagnostic tool for fasciolosis. FECs do, however, have some drawbacks. They are subject to false negatives as fluke eggs can remain sequestered in the gallbladder for some time before excretion and after treatment leading to undiagnosed early infections of *F. hepatica.* Only the adult egg-producing fluke (aged > 10 weeks) can be detected using FEC, the more damaging immature flukes can remain undiagnosed using this test (Andrews,1999)

Confirmation of infections post-mortem are performed routinely on animals submitted to disease surveillance centres. Liver investigations after death can be advantageous for disease surveillance and can encourage farmers to change their management techniques. From an economic and welfare perspective, confirmation of fasciolosis after death is not advantageous. A dead animal is not profitable to a farm and there is also a cost associated with the post-mortem, however the diagnosis is unequivocal and can be informative nonetheless. Both adult and juvenile fluke can be present at one time both inflicting damage to the liver (Behm & Sangster, 1999). Again, liver inspections after slaughter are performed routinely and the data collected from these is used to inform farmers and add to research in to the distribution and effects of liver fluke around the country (Innocent et al*.,* 2017; Mazeri et al., 2017).

Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect antibodies and antigens of *F. hepatica* in serum and are used routinely to diagnose exposure to infections of *F. hepatica* in livestock (Langley & Hillyer,1989; Zimmerman et al*.,* 1982). Detection of anti-Fasciola antibodies in serum is advantageous in the determination of infection 4-8 weeks post infection but is not suitable for the indication of a present infection in animals over one year old. Results from serum antigen ELISAs can indicate current infection and detect infection 1-2 weeks post-infection, albeit with limited sensitivity (Gordon-Gibbs, 2014). Another disadvantage of serum ELISAs is the collection of blood samples which is invasive and inconvenient for farmers, and little practised, certainly by sheep farmers. Biochemical assays for the measurement of liver enzymes gamma-glutamyl transferase (GGT) and glutamate dehydrogenase (GLDH) can determine bile duct and liver damage caused by fluke infection. Similarly, to some ELISAs, biochemical assays are not suitable for the detection of fluke in pre-patent infections (Gordon-Gibbs, 2014). The relatively recent coproantigen ELISA (cELISA) detects liver fluke antigens in faeces, which can be easily collected from farms and this test is effective at detecting low burdens of fluke, as early as four weeks post-challenge (Gordon-Gibbs, 2014).

DNA-based methods of detection are not routinely used to diagnose infections but both polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) are used as diagnostic tools in research. LAMP has greater resistance to inhibitory factors than PCR and has the potential to be applied in field situations for the detection of *F. hepatica* stages on the pasture, particularly metacercariae. Currently, these methods have only been applied to the detection and diagnosis of *F. hepatica* in live animals. The further development of these methods for the identification and quantification of liver fluke risk in the environment would help to identify disease risk in space and time to inform avoidance strategies and facilitate targeted disease control.

Loop-mediated isothermal amplification, or LAMP, is a molecular technique offering an alternative method of DNA amplification, aside from PCR, that is rapid, sensitive and specific (Notomi et al., 2000). Developed in 2000, this technique is able to amplify DNA under isothermal conditions utilising the activity of Bst polymerase instead of the classic Taq polymerase used in PCR (Notomi et al., 2000). The benefits of using LAMP over PCR include the ability of this method to work under a constant temperature (typically ~60°C) thus removing the need to use costly thermo cyclers (Notomi et al., 2000). The use of Bst has granted this technique greater resistance to inhibitors compared to PCR allowing the amplification of DNA from crude DNA samples (Melville et al., 2014; Sriworarat, et al., 2015).

The unique characteristics of this technique enables its possible application ‘in the field’ where it may offer a powerful diagnostic tool for farmers. LAMP assays have been developed to detect *F. hepatica* from faecal samples of naturally infected sheep, 7 weeks post infection. It took the LAMP assay 70 minutes to detect the *F. hepatica* DNA target compared to PCR which took 3 hours (Martínez-Valladares and Rojo-Vázquez, 2016). The LAMP and PCR assays used by Martínez-Valladares and Rojo-Vázquez (2016) had a limit of detection of 1 x 10^{-3} ng. A more sensitive assay was developed by Ai et al., (2010) with the ability to detect *F. hepatica* DNA at 1x 10^{-5} ng and this assay had no cross reactivity with *F. gigantica* or any other closely related trematode species (Ai et al., (2010).

The ability of LAMP to detect *F. hepatica* DNA from faecal samples, which can contain a number of inhibitors including starch, cellulose and bile salts known to inhibit PCR, is very promising for the proposed amplification of *F. hepatica* targets extracted from metacercariae in pasture samples. The high sensitivity of both LAMP is also promising when considering the small amount of *F. hepatica* DNA there is likely to be within a pasture sample.

1.7 Fluke Forecasting

The incidence of fasciolosis varies widely year to year, from farm to farm and from field to field. Particularly bad years involve many farms within large areas of the UK being affected, but the following year, incidence of fasciolosis may only be minimal. Long standing knowledge of the association between wet and warm summers with high risk years has allowed for the creation of liver fluke prediction models. These models fulfil the purposes of forecasting fluke risk, namely: establishing the status of fluke risk in the surrounding area of a farm, whether that predicted risk will be average, high or low for that year and determining times of highest metacercarial challenge on grazing fields (Charlier et al., 2014).

The first widely used method for the prediction of fluke risk in the UK was developed by Ollerenshaw and Rowlands, 1959. This method was originally developed for the island of Anglesey, but was later extended to include England, Wales and France (Ollerenshaw, 1971). Primarily, the prediction model was developed as an early warning system, allowing farmers time to remove stock from high risk areas, with the aim of preventing or limiting the ingestion of metacercariae by livestock. This early warning system was developed as a way of giving farmers an indication of the predicted level of disease incidence, thus benefiting the farmers further. Predictions from forecasts early in the season could reduce the pressure put on farmers to perform the challenging and time-consuming task of draining grazing fields, especially if the predicted incidence was low.

It was already well established that moisture and temperature directly affected snail survival, snail habitat and the lifecycle of *F. hepatica.* Ollerenshaw and Rowlands (1959) investigated correlations between climatic data and data on the prevalence of fasciolosis as a method of developing an accurate model.

Temperature limits the lifecycle and activity of both *G. truncatula* and *F. hepatica*. Below 10°C, miracidia do not hatch from liver fluke eggs and cercarial shedding from snails does not occur; from this is was deduced that in the months where the temperature rarely exceeds 10°C there is very little risk of fasciolosis (Kendall & McCullough, 1951, Ollerenshaw & Rowlands, 1959). Thus, temperature could define the starting point of development of miracidia, the replication of internal stages within the snail and the shedding of cercariae onto pasture. From this, the traditional disease cycle was established - egg hatching in early summer, to the continual infection of snails throughout July and August and cercarial shedding in the late summer and early autumn, culminating in cases of fasciolosis in the winter.

Wet weather during June and July triggers a rise in snail populations, the hatching of *F. hepatica* eggs and the expansion of snail habitat. It was also understood that the continuation of these weather conditions favoured the survival of the snails, allowing them to grow bigger, thus leading to heavier *F. hepatica* infections (Ollerenshaw & Rowlands, 1959).

A high incidence of fasciolosis was expected from summers where the climatic conditions favoured the survival of the snail i.e. rainfall was particularly high and temperature warm.

Although a correlation between rainfall and incidence of fasciolosis was understood, simply measuring rainfall was identified as a poor indicator of favourable moisture conditions for both snails and *F. hepatica* (Ollerenshaw & Rowlands, 1959). The rate of evapotranspiration (the movement of water from the earth to the atmosphere by evaporation and transpiration) was identified as a better measure. Mean rainfall measurements were deemed not to be an accurate representation of moisture levels for the whole month. A month could be wrongly classified as ‘wet’ if a high rainfall is recorded on a particularly wet day, even in a mostly dry month. A wet month could inversely be identified as a ‘dry’ month because there was light rainfall most days and the total monthly rainfall overall was low, but the level of moisture on the pasture remained constant.

To minimise this potential error, a ‘wet’ day was alternatively defined as rainfall exceeding the rate of transpiration and a ‘dry’ day as transpiration rate exceeding the amount of rainfall.

$$Mt = n\left(\frac{R}{25.4} - \frac{P}{25.4} + 5\right)$$

Ollerenshaw Index

The National Animal Disease Information Service (NADIS) still uses the ‘Ollerenshaw index’ method of forecasting and provides farmers with monthly regional predictions (NADIS, 2018). Despite the continual use of this method over the years, its application outside the UK and Ireland has not been quite as successful. The method requires extensive climate measurements; this is beyond the capabilities of most weather stations which may only record temperature and rainfall (Malone & Yilma, 1999).

Ross (1970) devised a simpler method for forecasting fasciolosis in Northern Ireland. This system assessed the number and distribution of “wet days” between the months of June and September. A “wet day” being defined as a day where 1mm or more of rain falls (Ross, 1970). Unlike the Mt value devised by Ollerenshaw and Rowlands (1959), data on the amount of rain falling on any given day could easily be gathered from any meteorological station without the need for specialised meteorological equipment. Ross argues that the Mt value does not account for changes in weather conditions and assumes consistency in weather. For example, a very dry June in Northern Ireland resulted in a reduced incidence of fasciolosis despite the conditions of the following months (Ross, 1970). From the data collected in June and early July, this system also allowed for a provisional early forecast.

The risk of fasciolosis forecast to farmers, by bodies like NADIS, is based on factors affecting the survival of *G. truncatula* and consequently could arguably be deemed a relatively crude method of determining the risk of fasciolosis at a farm and field level. A study conducted by Charlier et al., (2014) in Belgium, compared information collected on the abundance of *G. truncatula* on farms and combined this with data from the climate, micro-environmental factors and the within-herd prevalence of *F. hepatica* to determine farm-level predictions of *G. truncatula* and subsequent risk of *F. hepatica.* Managerial factors were also considered in relation to the spatial distribution of *F. hepatica.* It was determined that the mowing of pastures, the proportion of grazed grass in the diet and the length of the grazing season were important factors and were able to describe the spatial distribution of *F. hepatica* with greater accuracy than a model only containing climatic and environmental factors (Bennema et al., 2011).

More arguments as to the unsuitability of both the Ollerenshaw index and the Stormont “wet day” (Ross et al, 1970) forecasting systems for predicting fasciolosis today include the knowledge that these were developed using weather data collected in the 1950s and 1960s. Although these prediction models are still used as the basis of forecasts today and some of the same disease patterns exist, the farming industry and the climate of the UK have changed considerably. These models are also based on factors which affect the hatching of eggs and the survival of snails with little information as to the survival of metacercariae on pasture.

Geographical Information Systems (GIS) are another example of how fasciolosis can be studied spatially in relation to geographical and climatic variables. McCann et al., (2010) used this method to describe the different levels of fluke infection in dairy farms between different postcode areas of England and Wales. Using weather and pasture data they were able to explain 70% of the differences in liver fluke prevalence on dairy farms as determined by bulk tank milk ELISA. However, significant differences were observed between farms within the same postcode area and this could not readily be explained by the information provided in the GIS system (McCann et al., 2010).

The presence of viable *F. hepatica* metacercariae on grazing pasture is the ultimate risk of fasciolosis to livestock. Estimations and predictions of fasciolosis could be tuned and refined by including information on the climatic conditions suitable for metacercariae shedding and viability. As fluke predictions are currently given on a regional basis this approach to liver fluke prediction would be particularly beneficial to individual farms.

Overall the current prediction of disease incidence remains unreliable and indirect; fasciolosis is a not a notifiable disease, there is no active surveillance or national data set which documents cases and forecasting fluke risk is performed on a relatively crude regional scale, using traditional seasonal patterns. Additionally, significant differences in weather and climate occur within areas that have been reported to have the same meteorological predictions (Kendall, 1970), so fluke risk tends to be farm and even field specific. Of all the models created to forecast fluke risk none have been successful in establishing fluke risk of a farm, the predicted level of risk or the times of highest level risk on grazing fields (Charlier et al., 2014). Alternative strategies, complementary tools and the inclusion of more surveillance data will eventually lead to more accurate prediction of fluke risk, thus allowing for the implementation of avoidance strategies and targeted sustainable disease control (Fairweather, 2011).

Obviously, each stage in the lifecycle of *F. hepatica i*s important to the continuation of the parasite. Climatic factors aside, fundamentally, a farm is not at risk of fasciolosis if livestock are not grazing or the intermediate hosts are not present on grazing fields. More directly related to the presence of metacercariae is the ecology of *G. truncatula.* Although the focus of this thesis is on factors influencing the viability and presence of metacercariae, it is impossible to discuss this topic without referring to *G. truncatula,* as metacercariae cannot develop without this intermediate stage.

1.8 Intermediate snail host

The inclusion of intermediate snail hosts in the lifecycle of digenean (two-host) trematodes is well documented. These snails act as vehicles for larval development and for subsequent dispersal of infective larvae (metacercariae) into the surrounding environment, where they could be ingested by suitable definitive hosts. *G. truncatula*, formerly known as *Lymnaea truncatula,* is the main intermediate host for *F. hepatica* in the UK and Europe. Other, less well known carriers of *F. hepatica* in the UK include: *Lymnaea stagnalis*, *Radix auricularia*, *L. palustris*, *Galba glabra* (Skuce et al., 2014) and *Omphiscola glabra* (Abrous, Rondelaud & Cabaret, 1999).

In other countries many *Lymnaea sp. snails* are implicated in the lifecycle of *F. hepatica* (Torgerson & Claxton, 1999). Recently *Radix peregra* was confirmed to act as an intermediate host in Ireland (Relf et al., 2009) and *L. palustris* and *L. fuscus* have been confirmed as intermediate hosts for *F. hepatica* in Sweden (Novobilský, Kašný, Beran, Rondelaud, & Höglund, 2013).

The fast rate of genetic adaptation on the part of *F. hepatica* has been linked with its adjustment to the environments of definitive hosts (Cwiklinski et al., 2015) and perhaps it is this trait that has allowed the parasite to utilise many snail species (Richards, 2016). Despite this, the importance of these other species in the spread of fasciolosis and their ability to shed viable metacercariae is not fully understood. Metacercarial shedding of *F. hepatica* by

O. glabra was witnessed to be significantly less compared to *G. truncatula,* even when harbouring a higher number of rediae and co-infect with, the rumen fluke, *Calicophoron daubneyi* (Vignoles et al., 2017). The implications and importance of *C. daubneyi* to the diagnosis of *F. hepatica* in the environment is discussed in section 1.10.

G. truncatula is a mud snail, and as the name suggests, usually populates wet muddy areas subject to temporary flooding (Figure 1.3) (Dreyfuss, Vignoles & Rondelaud, 2015). These snails are not fully amphibious and will move in and out of water throughout the day, where they can feed on algae and any other vascular plants, preferring drier areas 4-5 cm above water at night (Dreyfuss, Vignoles & Rondelaud, 2015). The success of *F. hepatica* while amplifying within *G. truncatula* is determined by factors that also affect snail survival, such as, climatic conditions, soil moisture, food availability and the presence of predators.

Soil moisture and soil disturbance are the two most important factors for the establishment of habitat for *G. truncatula* (Moens, 1991). Snail populations rise significantly during the period between June and September with peak populations in July (Hourdin et al, 2006). This coincides with the driest time of year which, in Europe, is typically around the month of July. Moisture impacts the feeding, reproduction and growth of these snails, with >70% humidity triggering the snails to aestivate (Moens, 1991). Snails will withdraw their bodies into their shells to reduce water loss and protect themselves from drying out; they can survive like this for as long as 4.5 months (Dreyfuss, Vignoles & Rondelaud, 2015).

High rainfall causes soil to lose structure making the it more susceptible to poaching (Moens, 1991). Poached areas of fields with many sheep and cattle hoof prints, are also common areas to find *G. truncatula.* These small indentations in the soil offer protection to the snails by providing them with a separate microclimate, sheltered from sunlight and predators (Richards, 2016). Drainage furrows and poached areas of fields are other common areas to find *G. truncatula* (Dreyfuss, Vignoles & Rondelaud, 2015). Soil moisture is also important for the movement of miracidia and cercariae and areas with high prevalence of fasciolosis are usually areas where the field capacity for moisture is exceeded during high risk times (Moens, 1981).

The main food source of *G. truncatula*, is green algae. This grows on moist ground, feeding on faeces of livestock and other mammalian species present in the environment. The uncovered soil of these temporary flooded areas is not suitable for many small organisms and *G. truncatula* takes advantage of this lack of competition by feeding on algae.

Figure 1.3 - *G. truncatula* photographed in situ, found on the edge of a man-made wader scrape in July 2017.

Shell size and number of miracidia that infect snails can also impact the number of metacercariae produced. Shell height had a significant effect on metacercarial production, with bigger shelled snails producing more metacercariae than small shelled snails (Vignoles, et al., 2010). The more heavily infected with miracidia the greater the production of metacercariae, with snails infected with 3 miracidia producing more metacercariae than snails infected with 1 or 2 miracidia (Vignoles, et al., 2010). Snails can be infected with more miracidia but the number of metacercariae does not necessarily increase as snail size must be great enough to support an infection of five rediae (Vignoles et al., 2010).

Co-infections of snails with *F. hepatica*, *C. daubneyi* and other trematodes adds another layer of complexity to the epidemiology of *F. hepatica.* In some cases, the presence of other parasite stages has been suggested to reduce the production of *F. hepatica* metacercariae. For example, co-infections of *G. truncatula* with the frog trematode *Hapometra cylindracea* and *F. hepatica* (Goumghar, et al., 2000). In other cases, it has been suggested to be beneficial for *F. hepatica*; the presence of other parasite stages weakens the defences of snails and then allows *F. hepatica* miracidia to penetrate the snail mantle (Abrous et al., 1998).

The ability of *F. hepatica* to infect multiple snail hosts expands the potential risk areas in the environment. Acidic soils are an example of unsuitable habitat for *G. truncatula* but *R. peregra* is able to tolerate this and was suggested as the source of fasciolosis in sheep grazing this type of land in Ireland (Relf et al., 2009). This poses a challenge for the effective control of *F. hepatica.* Where the application of drainage might be useful for the control of *G. truncatula*, the same approach may not be applicable for the control of other snails.

1.9 Metacercariae

Metacercariae, or fluke cysts, are defined as the encysted, dormant, infective stage, to the definitive host, of digenean trematode parasites. Grazing animals become infected with *F. hepatica* after ingestion of pasture infected with *F. hepatica* metacercariae. Unlike the other environmental stages of *F. hepatica,* metacercariae are not required to seek out a new host or replicate. Their sole role, in the life-cycle, is to stay encysted on plants awaiting the arrival of the definitive grazing host and to remain resistant to environmental conditions in order to be capable of infecting their new host. Many factors contribute to the availability and viability of metacercariae in the environment. Understanding these factors is paramount to further understanding the epidemiology of *F. hepatica* and determining risk of fasciola infection to livestock.

1.9.1 Availability

Cercarial shedding is the process of cercariae leaving the body of the snail in search of a place to encyst. The availability of metacercariae is directly related to the shedding of cercariae as it is the encystment of cercariae that results in metacercariae on pasture. Understanding the mechanisms and conditions of cercarial shedding offers another opportunity to understand metacercarial availability and in turn another determinant of liver fluke risk.

Mature cercariae do not exit the snail all at once but in several bursts or waves. Shedding of *F. hepatica* cercariae and metacercarial dispersal by *G. truncatula* is discussed by Hodasi (1972). It is understood that the number of cercariae that will emerge from individual snails varies considerably. The average number reported by Hodasi (1972) was 594 cercariae per snail, with a range of 4-1789 (Hodasi, 1972). The presence of rediae, both mature and immature, within the snail results in the production of cercariae at different times. Mature cercariae will remain in the body of the snail until such triggers for their passive emergence occur. This results in a staggered release of mature cercariae (Kendall & McCullough, 1951). Although appearing to be an inefficient method of dispersal, it is thought that the parasite uses this method to guarantee that cercariae are released over the lifetime of the snail, and allow for some metacercariae to avoid adverse weather conditions (Hodasi, 1972).

Certain conditions are required for cercarial shedding. The essential conditions: snails must be in water, fresh water in particular, the temperature must be 10°C or above and a change in climatic conditions from low to high moisture (Dreyfuss et al., 2015).

The requirement of water for the activation of cercarial shedding is understandable, given that cercariae use their tail to swim and migrate to potential excystment areas. It was demonstrated by Kendall and McCullough (1951) that cercarial emergence was not influenced by light or darkness but could be induced by moving snails from a ‘dry or watery habitat’ and placing them in fresh water. The increased movement of the snail triggered by this change in habitat is thought to result in the passive shedding of mature cercariae from the body as the mantle walls of the snail contract. The demonstration of cercarial shedding by moving snails to fresh water was related to the laboratory management of snail colonies. The activation of cercarial shedding by fresh water mimics the environmental conditions where summer rain showers would activate the snails, consequently triggering the shedding of cercariae (Kendall & McCullough, 1951). It was also argued that this action allows for greater accessibility of the encysted metacercariae to grazing livestock. Temporary flooding within the microclimate of the field floor allows cercariae to access vegetation: the more rain that falls, the higher up the vegetation the cercariae can encyst. As rain evaporates and drains away this leaves the encysted metacercariae in advantageous positions to be consumed by grazing livestock (Kendall & McCullough, 1951).

The availability of metacercariae on pasture gives an indication as to where and when the highest level of risk might occur. Key to the availability of metacercariae on pasture is the presence of *G. truncatula* and grazing livestock within the surrounding environment. Without the presence of either one of these, there is no risk of fasciolosis. The presence of metacercariae on pasture suggests that climatic conditions are suitable for continuation of the lifecycle. For the production of metacercariae, it is essential that miracidia hatching from eggs find and infect snails, and the development of intra-snail stages must also be sufficiently timely so that mature cercariae are produced before the end of the lifespan of the snail. Environmental conditions can negatively impact this process, leading to failure in the production of infective metacercariae. In Iceland, where both *G. truncatula* and grazing animals such as sheep exist, fasciolosis is not an issue as the environmental conditions do not allow for timely development of the intra-snail stages despite there being probable introduction of *F. hepatica* to Iceland through the movement of livestock (Richards, 2016).

Cercariae have been shown to have a preference in excystment location. They prefer the green lower surface of leaves to encyst as demonstrated by higher numbers of metacercariae on the green parts of leaves compared to the brown or dead leaves (Hodasi, 1972). In doing so, it is thought that there is more chance of the metacercariae being eaten when on the green parts of plants as these would have better nutritional value for grazing livestock over dead leaves. Preference of plant type by cercariae could also dictate the availability of metacercariae. Out of nine plants commonly found in the environment of *G .truncatula*, *Juncus sp.* (rushes) had the highest percentage of encysted metacercariae (Pecheur, 1967). It was thought that the texture of the plant wall allowed for strong attachment of the cyst walls (Pecheur, 1967).

There is very little published information pertaining to the numbers and distribution of metacercariae on pasture during a typical high risk year. Obtaining data on the numbers of metacercariae on farms and fields for any given year would be highly valuable information for better understanding of *F. hepatica* epidemiology. Outbreaks of liver fluke have been determined by the identification of these stages on the pasture. The Netherlands Veterinary Services routinely use cellophane rafts for the collection of metacercariae from pastures to determine the level of infection risk to livestock (Gaasenbeek, Over, Noorman, & de Leeuw, 1992). This is particularly suitable for the Dutch system, as it takes advantage of the flooded nature of the grazing land. However, it is less suitable to pasture-based UK livestock systems. The same dikes and dams used to protect The Dutch system from flooding is not required on most of the UKs grazing land as it is elevated higher above sea level.

1.9.3 Viability

The viability of metacercariae on pasture dictates the level of risk to grazing livestock. The general consensus is that metacercariae are immediately infectious after encystment. However, fasciolosis did not occur in mice that were dosed with freshly encysted metacercariae but did occur in mice dosed with 24 hour old metacercariae (Boray, 1963).

Climatic temperature and moisture availability are two key determining factors of metacercarial survival (Andrews, 1999). Unlike the development and survival of eggs and snails in relation to temperature, the determination of metacercarial survival in relation to temperature is much more complicated (Rapsch et al., 2008). Between 2-5°C, 10% of metacercariae will survive a year (Boray, 1963). 100% of metacercariae will survive 6 months between 12°C -14°C (Boray, 1963 and Rapsch et al., 2008). The importance of moisture becomes more relevant to metacercarial survival when considering temperatures associated with the summer months, 20-30°C, in Europe. When stored in water at 20°C for 14 days, 90% of metacercariae were determined to be alive by microscopical examination and all experimentally challenged mice developed disease (Boray & Enigk, 1964). When the relative humidity was 75-80%, only 5% of metacercariae were deemed to be viable, and no mice developed disease after 14 days at 20°C. This highlights that metacercariae are highly susceptible to desiccation and further emphasises the importance of adequate moisture on pasture to ensure the longevity of viable metacercariae overtime.). Other key factors for metacercarial survival on pasture might include mechanical, chemical or bacterial infection after degradation of the cyst wall (Dixon, 1965). Metacercariae are vulnerable to all these factors whether encysted on fresh grass or within a preserved forage.

The morphological structure of *F. hepatica* metacercariae is important to their survival in the environment and was first described by Thomas, (1883). An oral sucker is situated to one end of the parasite contained within the cyst and the ventral sucker, of similar size, is situated just off centre. Flame cells are also prominent structures easily spotted when viewing viable cysts under a light microscope. These clusters of bright spheres group into two granular masses on either side of the ventral sucker along the length of the cyst body. Flame cells are part of the fluke’s excretory network, situated at the end of tubules they act much like a pair of kidneys (Lal, 2007). Waste material diffuses into the flame cells and this builds up during quiescence and thus creates the bright spherical structures. The movement of cilia within the flame cells and tubules allows for the ejection of this material during excystment, and it is this ‘flickering’ movement that inspired the name - flame cells (Bennett, 2013 and Martin & Hine, 2008) Metacercarial viability can be crudely determined by the presence of flame cells within the metacercariae during visual inspection under a dissection microscope. The morphological differences between viable and non-viable metacercariae are depicted in Figure 1.4.

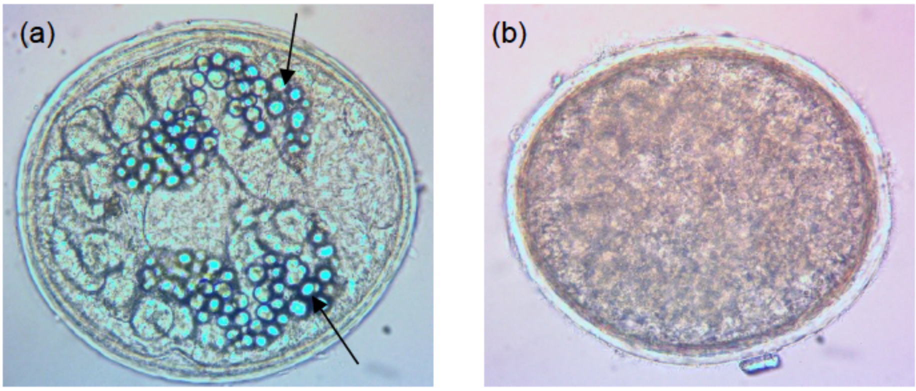

Figure 1.4 - Viable (a) and non-viable (b) *F. hepatica* metacercariae (Gordon-Gibbs, 2014). Viable metacercariae have clearly defined flame cells (arrows) and they are also apparent in newly excysted juveniles. Flame cells cannot be identified in non-viable metacercariae

The passage of metacercariae through laboratory animals is a long-standing method of understanding metacercarial viability and fasciolosis risk. The collection of adult liver fluke from liver post-mortem acts as the determining factor of infectivity. Microscopic examination of metacercariae is another method used in past research. However, the accuracy of demining metacercarial viability from visual inspection was brought into question when 90% of metacercariae determined to be viable under the microscope did not cause disease in mice (Boray & Enigk, 1964). Upon reflection, the authors determined that microscopic analysis of metacercariae was not a suitable method to determine viability of metacercariae.

Excystment assays are another established method for the determination of viable metacercariae (Tielens et al*.,* 1981; Hernández-González et al*.,* 2010; Garcia-Campos et al*.,* 2016). These assays mimic the conditions experienced inside the host animal triggering the emergence of a juvenile fluke from the metacercarial cyst wall. Observation of this process removes the subjectivity of determining viability from microscopic examination. Also, unlike the use of laboratory animals, excystment assays are performed *in vitro* and do not require sacrifice of animals. This is an advantage for research projects as the use of laboratory animals can be expensive and requires previously ethical permission. Other advantages are that excystment assays require only a few reagents and equipment to be performed and results can be obtained in a matter of hours. These attributes of the excystment assay make it a good method for the determination of metacercarial viability recovered from pasture.

There are many excystment assay protocols in the literature, differing in methodology, regents and efficacy. Some assays are designed to be performed in a laboratory setting, focused on the collection of the juveniles for further research studies such as proteomic analysis and migration behaviour (Garcia-Campos, Baird, & Mulcahy, 2016; Hernández-González, et al., 2010).

Currently, there does not exist an excystment method that could be used to determine the viability of metacercariae collected from pasture in an ‘on-farm’ situation. C. E. Bennett, in personal communications, suggested using a very simple method of excystment. This method can be performed inside Eppendorf tubes and requires no specialised equipment or expensive media. The simplicity of this method indicates that it has the potential to become a test that could be potentially translated into an ‘on-farm’ test. Rapid on-farm and field-specific assessment of fasciolosis risk allows for practical advice to be given to farmers in order to avoid and sustainably protect their stock from fasciolosis.

With this type of assay, data could be gathered on a country-wide basis; information on the viability of metacercariae on pastures throughout the year. Potentially, this information could help generate more accurate understanding of *F. hepatica* epidemiology and more accurate forecasts in the future.

To develop a test for the detection of *F. hepatica* metacercariae on pasture, there must be acknowledgment of the presence of other fluke species in the environment. The DNA of other trematode species could be amplified by the primers developed to detect *F. hepatica* when testing samples taken from *G. truncatula* or from pasture samples. The rumen fluke, *C. daubneyi,* is a current example of a fluke species that has relevance to the diagnosis of *F. hepatica* in the environment.

1.9.2 Diagnosis of metacercariae on pasture

There is an existing technique for recovering metacercariae from pasture which would be suitable for the environment encountered on farms in the UK. The Ministry of Agriculture Fisheries and Food (MAFF) published this method in 1986. It involves herbage samples being liquidised and strained before the addition of sulphuric acid to reduce particle size and the removal of air bubbles to allow the cysts to sink before viewing them under a microscope (Ministry of Agriculture, 1986). The MAFF technique boasts an average recovery rate of 90%; however, this technique is not used routinely in the UK because it is labour intensive and has significant Health and Safety concerns with the use of dangerous chemicals.

There are many other techniques which include the use of detergents and or mechanical separation of metacercariae from plant material and, depending on its purpose, each technique differs in the methodology applied and reagents used. Despite the existence of these methods, none are used routinely for the determination of metacercariae on pasture. The reasons behind this may be because they are not easy to perform, are potentially hazardous, require skill and copious amounts of time to identify metacercariae from plant material.

Employing molecular techniques for DNA detection would offer an easier, less hazardous, and accurate method of detecting *F. hepatica* from field material. These tools could be used in conjunction with the isolation and recovery of metacercariae in order to understand where metacercariae are and the level of viable infectious challenge on pasture. Theoretically, these tools could be used to pinpoint exact areas of fields where *F. hepatica* metacercariae are present. Not only can farmers make informed decisions as to the control of fasciolosis on their farms but any data collected can be fed back into research and a more detailed and accurate understanding of *F. hepatica* epidemiology.

1.11 Thesis aims

Fasciolosis, caused by *F. hepatica,* is a significant health and welfare problem for the livestock industry in the UK and beyond. The epidemiology of this parasite has changed in recent years and resurgence in cases has been attributed to many factors. New diagnostic tools are required to accurately determine the metacercarial challenge to livestock so that informed decisions can be made, and avoidance strategies implemented. The thesis aim is to investigate methods for the identification of viable *F. hepatica* metacercarial challenges to grazing livestock. To achieve this light microscopy and the C. E. Bennett method of excystment are compared as methods for determining metacercarial viability (Chapter 2). Methods to recover metacercariae from pasture are evaluated and demonstrated in both the laboratory and in the environment (Chapter 3). Recovery techniques are applied to evaluate the risk of fasciolosis from agri-environment schemes specifically, wader scrapes (Chapter 3). Recovery and viability assays are applied to understand the longevity of *F. hepatica* metacercariae at different pH associated with silage fermentation and in silages of different qualities (Chapter 4). Finally, both PCR and LAMP are demonstrated and compared in their abilities to detect *F. hepatica* metacercariae from samples of DNA extracted from grass spiked with *F. hepatica* DNA and from grass spiked with metacercariae (Chapter 5).

Chapter 2: Visual and functional methods of determining *F. hepatica* metacercarial viability

2.1 Introduction

Metacercariae are the infectious, dormant stage of the *F. hepatica* life cycle. Viable metacercariae pose a disease risk to livestock who graze contaminated grass or forage. Metacercarial viability is defined by the ability of a metacercaria to survive on the pasture or in forage long enough to be ingested thus, allowing for the continuation of the life cycle. The time limit of metacercarial viability grazing pastures or in forages is difficult to determine. There exist many factors that can influence the amount of time metacercariae remain viable. Metacercariae are thought to be capable of surviving encysted on grass for several weeks provided that there is sufficient moisture present in the environment to prevent death by desiccation (Leuckart, 1886; Meek & Morris, 1979; Olsen, 1947

It is becoming increasingly difficult to manage fasciolosis on farms. Populations of fluke resistant to anthelmintics, particularly the drug with the widest range of effect of fluke stages, triclabendazole, makes it difficult to effectively treat animals with fasciolosis. Resistant populations of fluke can result in a cycle of pasture re-infection and a prolonged issue with fasciolosis on farms. Financially for farmers this can have a serious economic effect (Sargison and Scott, 2011). Climate change is another increasingly important risk factor. Shorter, milder winters and wetter summers has allowed for the increased survival of *F. hepatica* life stages on pasture and the expansion of habitat suitable for *G. truncatula* (Mitchell, 2002; Howell, et al., 2015). In the absence new anthelmintic drugs or a vaccine to protect against *F. hepatica* and with the effects of climate change predicted to expand and worsen the problem of fasciolosis in the UK (Fox et al*.,* 2011) the importance of disease avoidance has become more significant.

Predictions of fasciolosis are key to avoidance of fasciolosis and prevention of outbreaks. Fasciolosis forecasts are already established regionally for the UK (NADIS, 2019). But the methods used are deemed relatively crude and do not suit the prediction of fasciolosis on a specific farm or field requirement. Understanding the factors which affect metacercarial viability on pasture is key to the development of accurate, targeted forecasting. Imperative to the development of accurate fluke forecasting are scientific methods which can effectively and easily determine metacercarial viability in the lab and from metacercariae collected from pasture.

The effect of salinity on metacercarial viability is not known but understanding it could confirm that salt rich areas in the environment are fluke risk free. Salt water merse is perceived to have low risk of fasciolosis by farmers. *G. truncatula* is seldom reported in tidal areas and is only found in waters of no higher than 5% salinity (Styczyncka-Jurewicz, 1966). Additionally, salinity levels compromise the development of *F. hepatica* eggs with 16% salinity the upper limit for the hatching of miracidia (Styczyncka-Jurewicz, 1966). There exists no data reporting the effect of salinity on metacercarial viability, however, the previous information may indicate that saltmarsh areas offer little risk of liver fluke to grazing livestock and that grazing saltmarshes might offer a potential method of disease avoidance (Minting & Seymour, 2016). Another advantage to grazing salt water merse is the creation of grass sward suitable for the habitat of natterjack toad, *Epidalea calamita*, Scotland's rarest amphibian (found only along the Solway coast). Farmers are paid annually to follow a grazing pattern on the salt water merse of the Caerlaverock National Nature Reserve (Scottish National Heritage, 2016). These toads can be found in saltmarshes or 'merse' in this reserve where they lay their spawn and utilize the surrounding coastal farmland to forage and hibernate (Minting and Seymour, 2016).

Determining the viability of metacercariae collected from pasture samples, particularly specific areas of interest i.e. salt water merse, would give an indication of the infective risk posed to livestock throughout the grazing season. Isolating and determining the viability of *F.*

hepatica metacercariae from pasture would provide valuable data on the survivability of liver fluke under specific climatic conditions. Metacercarial viability data can be assessed in terms of the preceding meteorological data from a particular farm of origin. This would give significant insight into the fluke life-cycle on a specific farm for a specific season. Meteorological data can indicate when the cercariae were likely to have been shed when backdated against the date of cyst isolation. The viability of the metacercariae isolated will indicate if the climatic conditions were suitable for their survival. Performing cyst recovery and isolation from pasture from many farms would generate more data for scientists who can develop better models for the determination of liver fluke risk across the UK.

Currently, there does not exist a routine method for determining the viability of metacercariae collected from pasture. Assessment of metacercarial viability is predominantly performed under laboratory conditions using cysts shed by laboratory reared snails. Over many years of research, there exist a number of visual or functional methods to measure metacercarial viability in the lab. In previous literature, metacercarial viability was determined by; establishment of infection in laboratory hosts (Boray & Enigk, 1964; Valero & Mas-Coma, 2000) *in vitro* excystment assays (Tielens et al*.,* 1981; Hernández-González et al*.,* 2010; Garcia-Campos et al*.,* 2016) and by visual inspection using light microscopy (Wikerhauser, 1960; Suhardono, Roberts, and Copeman, 2006).

Pertinent to the determination of fasciolosis risk on a farm or field level is the speed of results. Light microscopy and excystment assays are two quick methods of determining metacercarial viability. The disadvantage of using animal models is the length of time it takes for the passage and establishment of liver fluke infections. Other draw backs of using animal models to determine metacercarial viability include the costs associated with rearing, housing and infecting animals, and the labour and skill required to post-mortem and process the animals. Using animal models is also not conducive to the 3R's (Replacement, Reduction and Refinement), an important part of animal ethics in scientific research (Graham and Prescott, 2015).

Light microscopy to determine metacercarial viability involves inspecting the morphology and integrity of metacercariae. Visually inspecting metacercariae for signs of deterioration gives a quick indication as to the level of viability in a cohort of laboratory reared metacercariae. This type of inspection is common and might be performed weekly as part of the regulatory maintenance of metacercarial cohorts stored in water for the purpose of research.

The subjective nature of visual determination of metacercarial viability makes it difficult to consistently determine metacercarial viability and can vary depending on the experience of the performing technician. Another, very simple, method of visually assessing viability incudes the staining of metacercariae with 0.1% toluidine blue then placing them on a hot stage (38°C) to initiate movement within the cyst (El-Sayad et al., 1997). Observation of the fluke moving within the cyst wall indicates that the metacercariae is viable. Although this method removes subjectivity, by emulating the ingestion of the metacercariae with temperature change, it does not demonstrate the ability of the metacercariae to excyst.

Excystment assays mimic the conditions of a host gut *in vitro* and are used as a functional method for determining metacercarial viability. Metacercariae require specific conditions in order to excyst. The physiology of *F. hepatica* metacercarial excystment was described by Dixon (1966). Excystment assays allow for the viability of metacercariae to be determined easily, rapidly (within a few hours) and relatively cheaply compared to *in vivo* or *ex vivo* methods. The use of excystment assays is advantageous for researchers because they do not require approval of an ethics committee, which is contrary to the use of mice or another laboratory model for the passage of liver fluke.

There are many variations of the excystment assay, differences in assay protocol depend on the nature of the work being carried out. Much of the research happening presently on *F. hepatica* is focused on the discovery and creation of new drug and vaccine candidates of which newly excysted juveniles (NEJs) are prime targets (Garcia-Campos, Baird, & Mulcahy, 2016; Hernández-González , et al., 2010). Excystment assays allow for isolation and collection of NEJ’s, much like the method developed by Tielens et al., (1981) which is able to incorporate 10,000 metacercariae and result in 60%-80% excystment rate and the use of an artificial gut wall allows for the collection of viable NEJ’s. The highest excystment rate found in literature for this project was 95%, achieved by Hernández-González et al., (2010). The excystment assay was based on one published by Smith and Cleggs (1981).

Both light microscopy and excystment assays allow for the determination of metacercarial viability quickly, this is key to the determination of fasciolosis on farms. In order to accurately diagnose fasciolosis risk of farms, reliable methods are required. Future development of these methods would ideally allow for ‘on farm’ testing. Thus also meaning that the methods used must be suited to these situations, which would lack the equipment and resources of a lab. Whilst researching excystment methods that might be suited to ‘on farm’ use, C. E. Bennett suggested, in a personal communication, his own excystment method. This method was developed by C. E. Bennett can be performed in an Eppendorf tube and requires only basic reagents and equipment whist also effectively creating the conditions suited for metacercarial excystment. The simplicity of this method meant that it had potential to become a test that could be potentially translated into an ‘on-farm’ test.

The aim of this Chapter was to compare the ability of light microscopy and the ability of the C. E. Bennett excystment method to determine metacercarial viability. These methods were demonstrated on metacercariae subjected to one well researched factor of viability, temperature and another less known known factor of viability, salinity.

2.2 Methods and materials

2.2.1 The effect of temperature on *F. hepatica* metacercarial survival

Experimental set-up

The longevity of metacercarial of viability and infectivity of metacercariae after exposure to temperatures experienced in the UK climate (0-14°C), is well understood. Metacercariae can survive a year when kept at 2-5° in moist conditions and 302 days when kept between 12-14°C in moist conditions (Boray, 1963). Above 20 °C the rate of metacercarial mortality increases; 90% of metacercariae were viable after 36 days at 20°C however only 80% of metacercariae were viable at 25°C, 36% at 30°C and 0% at 35°C when incubated for the same amount of time (Boray & Enigk, 1964). The following temperature experiment was set up to determine the suitability of two methods in the determination of determine metacercarial viability. As the effect of temperature on metacercarial viability is well understood it was chosen as a factor to compare the results of both light microscopy and the C. E. Bennett excystment assay when assessing metacercarial viability. The results of both methods were compared to previous finding in the literature.

4°C was selected for the experiment as this is the routine temperature for storing laboratory metacercariae in fridges and a prior understanding that a high percentage of metacercariae remain viable for a long time at this temperature. 25°C was selected as the other temperature for the experiment as this represents the upper limit of temperature tolerance for metacercariae and because UK temperature rarely exceeds 25°C.

Metacercarial viability was evaluated after 15 days and after 30 days of exposure to the two temperatures. These times were selected based off the experimental design of Boray and Enigk (1964) who assessed metacercarial viability with similar time intervals.

300 *F. hepatica* metacercariae (strain 'Italian', Ridgeway Research, UK), were used in this experiment. 150 metacercariae were designated to each temperature group. In each group,

60 metacercariae were excysted; 30 metacercariae at 15 days and 30 metacercariae after 30 days. The remaining 90 metacercariae were evaluated for viability using light microscopy; 30 metacercariae at 15 days, 30 metacercariae after 30 days and a further 30 metacercariae to be evaluated, by light microscopy, at both 15 and 30 days. This last sub-group hoped to measure the effect of repeat handling on metacercarial viability by light microscopy.

2.2.2 The effect of salinity on *F. hepatica* metacercarial survival

Experimental set-up

The effect of salinity on metacercarial viability is unknown. This experiment was designed to determine the viability of metacercarial under different salinity conditions whilst also evaluating the ability of light microscopy and the C. E. Bennett method to determine metacercarial viability. Metacercariae were exposed to different concentrations (%) of Sodium Chloride (NaCl). The salinity concentrations were selected to best emulate the salinities that might be present on salt water merse. Similarly to the temperature experiment the viability of metacercariae was assessed after 15 days and 30 days.

600 *F. hepatica* metacercariae (strain ‘Gloucester’, Ridgeway Research, UK), were used in this experiment. Saline solutions of NaCl were prepared with water to the following percentages - 2.5, 5, 7 and 10%. For each saline condition 30 metacercariae were assigned. A control group of 30 metacercariae were placed in water. This set up was repeated to give two sets of metacercariae at different percentages of salinity, one set to be excysted after 15 days the other after 30 days.

2.2.3 Determination of metacercarial viability - Light microscopy

Under a dissection microscope, the morphology of metacercariae was evaluated. The encysted metacercariae which were morphologically intact were deemed viable. Those cysts with slight damage to the outer cyst wall were deemed non-viable. Non-granular (flame cells) or obvious morphological disintegration of the juvenile fluke was another determinant of non-viable metacercariae. Example images of viable and non-viable metacercariae as obvious by light microscopy can be seen in the results Figure 2.1

2.3.4 Examples of metacercarial viability

Image examples of metacercarial viability as determined by excystment and some obvious example of viability as determined by light microscopy are shown in Figure 2.1

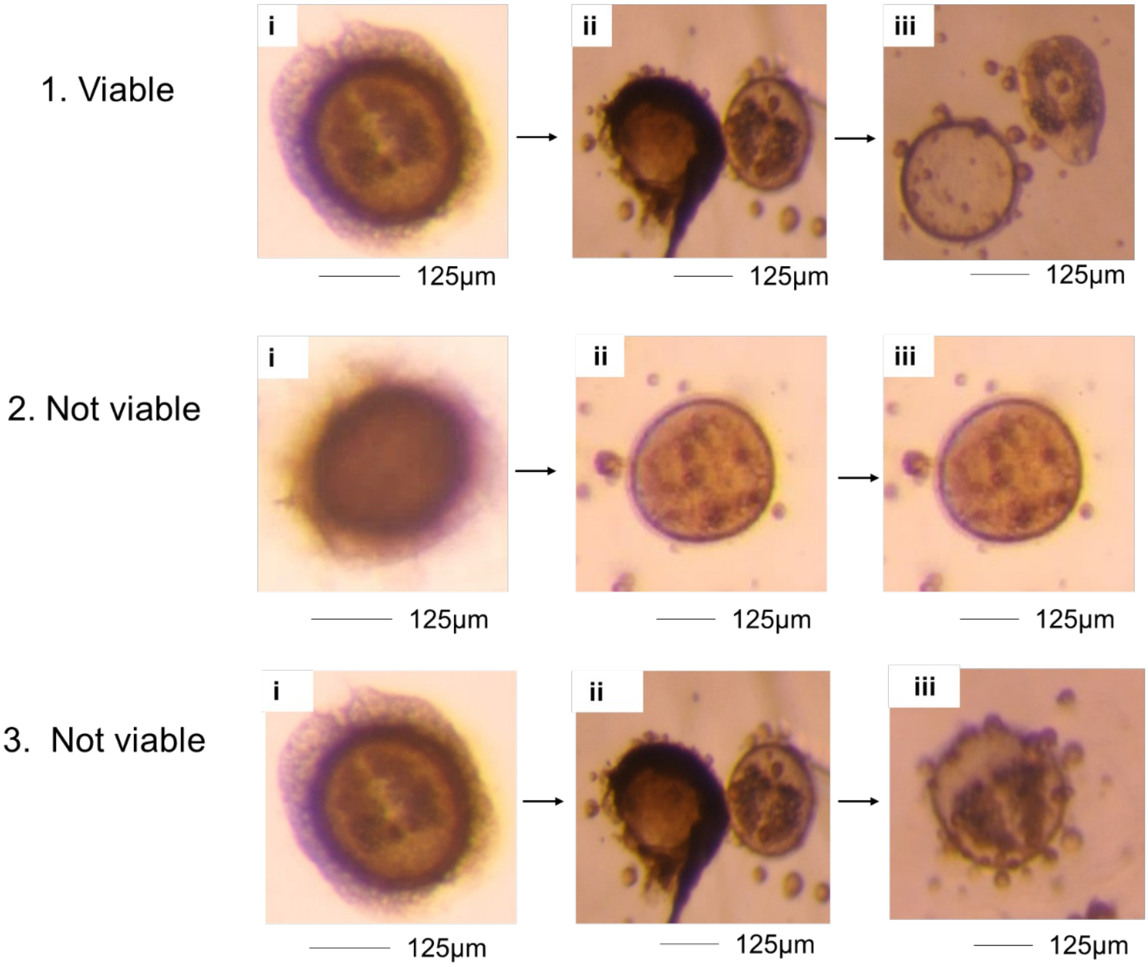

Figure 2.1 – Examples of metacercarial viability from the results of an excystment assay. 1. Viable (i) Metacercaria with both cyst walls (inner and outer) and juvenile intact flame cells clearly visible, (ii) Metacercaria inner cyst with outer cyst wall removed, (iii) Newly excysted juvenile fluke next to inner cyst wall. 2. Not viable, example also obviously determined by light microscopy (i) Metacercaria with decaying outer cyst wall, flame cells not visible, (ii) Inner cyst with outer cyst wall removed, (iii) metacercaria failed to excyst, the visible deterioration of the juvenile fluke also confirms this metacercaria is not viable. 3. Not viable (i) Metacercaria with both cyst walls (inner and outer) and juvenile intact flame cells clearly visible. (ii) Metacercaria, inner cyst with outer cyst wall removed, (iii) Same metacercaria, by light microscopy the lack of deterioration and visible flame cells would suggest that this metacercaria was viable but it failed to excyst.

2.2.4 Determination of metacercarial viability – Excystment assays

<u>Bennett method</u>

Metacercariae under the temperature and salinity study were excysted using the method advised by Dr Clive Bennett (University of Southampton). Metacercariae were checked for morphological soundness and the outer cyst wall removed by gently applying pressure with a cocktail stick. This process is supposed to mimic the effect of salivary enzymes and host chewing. Cysts were then transferred into excystment media containing 10% (w/v) sheep bile (Dryden Farm, University of Edinburgh, UK) in PBS with 3% $NaHCO_3$ and incubated in a water bath for two hours at 37°C. Juvenile fluke were viability scored by examination under a dissecting microscope. Morphologically intact and motile flukes were considered viable. Disintegrating flukes still within the cysts were considered dead.

Modified Hernández- González et al., (2010) excystment method

Excystment results achieved using the C. E. Bennett method were poor. Another excystment method was selected from the literature based on its high excystment rate. I received training in the excystment assay described in Hernández-Gonzalez (2010) under the direction of Dr M. Siles-Lucas (Institute of Natural resources and Agrobiology of Salamanca). Two assays were staged to demonstrated the performance of this excystment method when using ovine and bovine bile. 2 groups of 100 *F. hepatica* metacercariae (strain ‘Miskin’, Ridgeway Research, UK), were used; 100 were allocated for the assay including ovine bile and 100 for the assay using bovine bile. Ovine (Dryden Farm, University of Edinburgh) and bovine bile (calf, unknown location) were supplied Dr Rob Kelly (University of Edinburgh).

Slight adjustments were made to make the method easier to perform with the equipment and resources available. Instead of bubbling carbon dioxide through cold water for 30 seconds a bottle of sparkling water was used.

Assays were performed *in vitro* at the same time. Activation media consisted of 1 ml of 0.2M sodium dithionite (Thermo Scientific) dissolved in 10ml of cold carbonated, distilled water (Highland Spring). This solution was incubated at 39ºC for ~15 minutes until a cloudy precipitate was formed. Activation of the metacercariae was achieved by incubating metacercariae in the activating media for 1 hour at 39ºC. Before the addition of emergence media, metacercariae were washed twice with warm distilled water. Metacercariae were then transferred into this medium containing 10% (v/v) bile, in 5ml of Hanks' balanced salt solution, and 1 ml of 30mM HEPES (Thermo Scientific). Metacercariae were incubated at 39ºC in the emergence media for a total of 5 hours, with the collection of juvenile fluke beginning after 2 hours.

2.3 Results

2.3.1 The effect of temperature on *F. hepatica* metacercarial survival

Regardless of temperature condition metacercarial viability was determined to be higher when light microscopy was used compared to viabilities determined by excystment assays. At 15 days, metacercariae incubated at 4°C were 77% viable when evaluated using light microscopy but only 23% were viable when evaluated using excystment (Table 2.1). Similarly, the viability percentage was higher when evaluated with light microscopy (30%) after 15 days incubation at 25°C compared to evaluation using excystment for the same incubation time (0%) (Table 2.2). The viability percentage was also much greater when metacercariae were incubated at 4°C compared to 25°C, regardless of the viability method used.

At 4°C, the viability of metacercariae was similar between the groups incubated for 15 days and 30 days (Table 2.1). Using excystment assays the viability drops only 3% from 15 days to 30 days. In contrast there is a more marked drop in viability when viability was determined by light microscopy; 20% drop in the 15 days between the two time points. In the group which had its viability observed twice by light microscopy there is a 3% drop over time.

Metacercarial viability was much lower when incubated at 25°C. None of the metacercariae excysted in the group incubated for 15 days but 13% of the metacercariae incubated for 30 days did excyst (Table 2.2). Viability was determined to be higher using light microscopy, 30% at 15 days and 53% viability at 30 days. Metacercariae checked for viability twice using light microscopy had a lower viability percentage after 30 days (13.3%) compared to 15 days (20%) when incubated at 25°C.

Table 2.1 – Metacercarial viability (%) as determined by excystment and light microscopy when incubated at 4°C for 15 and 30 days

4°C	**Excystment**	**Light microscopy**	
15 days	23	77	
30 days	20	57	
15 vs 30 days	-	63	60

Table 2.2 – Metacercarial viability (%) as determined by excystment and light microscopy when incubated at 25°C for 15 and 30 days

25°C	**Excystment**	**Light microscopy**	
15 days	0	30	
30 days	13	53	
15 vs 30 days	-	20	13

2.3.2 The effect of salinity on *F. hepatica* metacercarial survival

Similarly to the results of the temperature experiment, metacercarial viability under different saline concentrations was always determined to be higher when using light microscopy over excystment assays.

Metacercarial viability under saline conditions, determined by excystment, was very low; ranging from 0-3%. The 3% viability determined by excystment assay was observed in the control group at 15 days and the group in 2.5% NaCl for 30 days (Table 2.3). Only 1% of metacercariae were able to survive 5% NaCl (determined by excystment) and no metacercariae were able survive NaCl concentrations above 5%.

The viability of metacercariae, as determined by light microscopy ranged from 53-77%. Viability remained very similar, regardless of NaCl concentrations, between viability of the groups determined at 15 and 30 days.

Table 2.3 – Metacercarial viability (%) as determined by excystment and light microscopy in different concentrations of NaCl for 15 and 30 days

% NaCl	**Excystment**		**Light microscopy**	
	15 days	**30 days**	**15 days**	**30 days**
Control	3	1	60	70
2.5	1	3	73	77
5	1	1	60	60
7.5	0	0	77	77
10	0	0	53	77

2.3.3 Modified Hernández-González et al., (2010) excystment assay

Excystment percentages were very high regardless of the species of bile used. When ovine bile was used the excystment percentage was 7% higher (Table 2.4).

Table 2.4 – Metacercarial viability (%) as determined by the modified Hernández- González et al., (2010) excystment assay comparing ovine and bovine bile

Bile	**Ovine**	**Bovine**
Excystment %	97	90

2.4 Discussion

The performance of light microscopy and C. E. Bennett excystment method to determine metacercarial viability was compared using metacercariae exposed to different temperatures (a well understood factor) and different salinities (a lesser understood factor of metacercarial viability). A modified version of the Hernández-González (2010) excystment method was also demonstrated using ovine and bovine bile.

Metacercarial viability was higher, regardless of method used, when metacercariae were incubated at 4°C compared to 25°C. This result is consistent with data in the literature describing the effect of temperature on metacercarial viability and from what has been observed personally in the laboratory. In the experiment assessing the effect of salinity on metacercarial viability there was an obvious difference in results between those metacercariae assessed using the C. E. Bennett excystment assay vs light microscopy. At 10% salinity, 0% of metacercariae excysted after 15 days compared to 53% of metacercariae estimated to be viable by light microscopy.

The results obtained from the temperature and salinity experiments are inconsistent. There was a clear difference in the viabilities of metacercariae determined by light microscopy compared to those determined by the C. E. Bennett excystment method. The percentage viabilities determined by light microscopy were always higher than the excysted counterparts. This was true for both the temperature and the salinity experiment. Inconsistencies were also evident when comparing the viabilities at different time points. Where an effect of time might be expected to be apparent in both experiments i.e. lower viability at 30 days compared to 15 days, the percentages viabilities were entirely opposite and contradictory. At 15 days the viability was expected to be higher than at 30 days because metacercarial viability decreases with age (Valero & Mas-Coma, 2000).

The unsuitability of the C. E. Bennett excystment assay is highlighted by the poor excystment percentages (3% and 1%) of metacercariae under controlled conditions. It is expected that relatively fresh metacercariae kept under controlled laboratory conditions should have a high level of viability.

The unreliability of the results from both experiments is highlighted further when comparing the excystment results obtained using the modified Hernández-González (2010) excystment assay. The viability percentages of control metacercariae which were not subject to any variables were not as high as the percentages resulting from the Hernández-González (2010) excystment assay. This assay was referenced to have a excystment percentage of 95% which is reflected in the results. The 90% excystment using ovine bile and 97% excystment using bovine bile from relatively new, lab reared metacercariae (cercariae shed a weeks prior to excystment) shows that this method works very well in controlled conditions. The metacercariae used in the temperature and salinity experiments were also lab reared (Ridgeway Research). The strains provided were subject to availability of metacercariae. It is not believed that the strain of metacercariae influenced the results. The quality of the commercially bought metacercariae should be high considering that these can be used to discover possible gene candidates for vaccine development (Dominguez et al., 2018). Despite confidence in the quality of commercially produced metacercariae the effect of different strains on the viability results cannot definitively be ruled out. Future work could explore the viability of different liver fluke strains by comparing the results of a valid excystment method or comparing the numbers of adult fluke recovered from infected model sheep. Researchers may be interested whether one strain has higher viability than another and whether strain viability is effected by time as they are passaged sequentially through snail colonies.

There is a tendency to overestimate the viability of metacercariae when using visual inspection to evaluate physical soundness of metacercariae. The inconsistency of the results gathered during the experiments performed for this chapter also highlight the unsuitability of light microscopy as a method for determining metacercarial viability. Figure 2.1 clearly shows how a visually intact metacercariae can appear to be viable but will not excyst. The number of metacercariae determined viable by microscopy can often be higher than those determined by excystment assays (Kim et al., 1998). This is further highlighted with reports of visually intact cysts that are unable to cause disease. Metacercariae exposed to -20°C were deemed to be alive when examined under a microscope but fasciolosis was not witnessed in mice that were infected with these same metacercariae (Boray & Enigk, 1964). It was reported that freezing metacercariae at -20°C did not kill the metacercariae but caused permanent and visually undetectable damage which rendered the cysts unable to cause disease (Boray & Enigk, 1964). Visual inspection of metacercarial integrity cannot be used as a reliable test of metacercarial viability. Although, it is still helpful for a quick rudimentary assessment of metacercarial death by trained laboratory technicians, as decaying metacercariae or desiccated metacercariae are easily discernible from morphologically sound metacercariae.

Replication of the C. E. Bennett excystment method in this chapter produced very low excystment percentages. The excystment percentages were low from what was expected, considering the age of the metacercariae and compared to the excystment percentages reported by other published methods. When considering the work of Dixon (1964) and comparing his findings to the C. E. Bennett assay used, a number of issues were identified that might explain why the assay did not work optimally. Firstly the method of metacercarial activation was not thought to be adequate. The activation stage of the C. E. Bennett method used a cocktail stick to remove the outer cyst wall. This process is supposed to mimic the effect of salivary enzymes and host chewing. The process of removing the cyst walls with a cocktails stick was a long, tedious and difficult process. The delicate nature of the metacercariae made it difficult not to accidently kill them by pressing two hard on the outer cyst wall. Other assays have similarly mimicked this process by instead placing the metacercariae between two sheets of glass and rubbing them together (Tielens et al., 1981). However, mechanically removing the outer cyst wall is not necessary to the activation of metacercariae. Activation of metacercariae is an active process triggered by an increase in carbon dioxide, reducing conditions and a temperature of ~39°C (Dixon, 1966).

The work of Dixon (1964) revealed that *F. hepatica* metacercarial excystment is not a passive process. Dixon argued that high carbon dioxide conditions found in the digestive tract of most mammalian hosts acts as a stimulus for many infective stages of parasites to exuviate their outer walls. Dixon also argues that the rare occurrence of anaerobic conditions in the environment has resulted in many parasitic organisms to evolve using this mechanism. Similarly, to other parasitic organisms such as coccidia and nematodes, Dixon suggests that the metacercariae are stimulated to produce enzymes which actively breakdown the cyst wall. Dawes (1961) also made this observation when investigating the migration of newly excysted juveniles.

Including the use of cocktail stick in the C. E. Bennett method to mechanically remove the outer cyst wall is arguably unnecessary and potentially damaging to the metacercariae. Other excystment assays, including Hernández- González et al., (2010) do not use a mechanical activation step.

With the knowledge that anaerobic conditions trigger the release of activating enzyme it was then thought that the assay was not adequately anaerobic. To emulate anaerobic conditions in the C. E. Bennett excystment assay, closed Eppendorfs containing metacercariae in excystment media were wrapped in cellophane. The conditions created may not have been sufficient to trigger the release of enzymes by the juvenile fluke to break down the ventral plug (the space in the cyst wall where the juvenile fluke excysts). To improve upon this it was suggested by C. E. Bennett that carbonated water could be used within the excystment media as a quick and easy way to emulate the conditions of the host gut. Hernández-González et al., (2010) method bubble pure CO_2 from gas canisters in to water in order to achieve metacercarial activation which requires the user to have access to canisters of CO_2. By using carbonated water, which can be bought from any supermarket, the cost and ease of creating adequate anaerobic contusions for this assay is greatly reduced. This suggestion, from C. E. Bennett was used to modify the Hernández- González et al., (2010) excystment method.

The modified Hernández-González et al., (2010) method of excystment was very successful when demonstrated using ovine and bovine bile. This assay included similar elements; redox potential, bile and temperature of around 39°C but the regents and assay design were entirely different to the C. E. Bennett method. Most notably, the Hernández-González et al., (2010) method incorporates the use of an activation and excystment media which follows the findings of Dixon (1964). For the C. E. Bennett method, metacercariae are exposed to all regents at once. By including an activation and excystment media, the Hernández-González et al., (2010) method of excystment more accurately portrays the natural process of metacercarial movement through the host gut.

Future repeats of the experiments performed in this Chapter would utilise the Hernández-González et al., (2010) method of excystment. This is a consistent scientific method but has yet to be tested statistically for reliability. Statistics were not applied in this Chapter because if was apparent that the methods use to determine viability were not reliable. A repeat study would incorporate statistics to and a reliable excystment method to accurately determine the effect of salinity on metacercarial viability.

Further development of the Hernández-González et al., (2010) method would focus on applying this tool for determining fasciolosis risk on farms from metacercariae collected from pasture. The use of carbonated water over canistered CO_2 to create suitable anaerobic conditions is one such modification. However, there are currently many other barriers that prevent the application of this tool to farms. Considerations such as equipment, reagents, technical training and the development of effective metacercarial recovery methods must be addressed first. In the absence of the required infrastructure for ‘on-farm’ testing focus should instead move to determine the performance of the Hernández-González et al., (2010) excystment assay on metacercariae collected from the environment. Grass samples could be processed for metacercariae and tested in lab as part of a nationwide surveillance scheme. The epidemiological data collected from something like this would improve upon the fluke risk models already provided.

2.5 Conclusion

The results of metacercarial viability when comparing light microscopy and the C. E. Bennett method of excystment to previously published data indicate that these are not ideal methods for determining metacercarial viability. The results of the temperature experiment did not align with the published data on metacercarial viability. Light microscopy produced variable results, often over-estimating the viability of metacercariae based on their morphological soundness. The C. E. Bennett excystment method resulted in underestimations of metacercarial viability at different temperatures. The excystment assay conditions of the C. E. Bennett method were not suitable for *F. hepatica* excystment as described by Dixon (1964). The Hernández-González et al., (2010) method of excystment was demonstrated and was successful when modified to use carbonated water. This assay produced percentage viability results that were expected when performed on laboratory metacercariae kept a 4°C, regardless of the species of bile used.

Future experiments would involve assessing the validity of the Hernández-González et al., (2010) as a method of excystment and determine its performance on metacercariae collected from the environment. Statistical analysis should be incorporated into future repeats of experiments on the effect of salinity on metacercarial viability.

Chapter 3: Detection of *F. hepatica* metacercariae on pasture/in the environment, with emphasis on the risk of fasciolosis associated with agri-environmental schemes

3.1 Introduction

Improved understanding of *F. hepatica* epidemiology is required to address and reduce the significant burden of losses to the livestock industry caused by fasciolosis. Development of existing and new environmental detection techniques and methods to predict disease risk is key to this. The National Animal Disease Information Service (NADIS) use a forecasting model based on the equation developed by Ollerenshaw (1971) called the ‘Ollerenshaw Index’. The rate of evapotranspiration, which is the combined movement of water to the air from soil (evaporation) and movement of water from plants (transpiration), is the base of this prediction model. Rainfall (R, mm/month) and temperature data gathered by the Met Office is used to determine the Mt value or monthly fasciolosis risk value. The Mt value, or the monthly fasciolosis risk value, is derived from rainfall (*R,* mm/month) minus transpiration (*P,* mm/month), multiplied by the number of rain days (*n)* (Ollerenshaw & Rowlands, 1959):

$$Mt = n\left(\frac{R}{25.4} - \frac{P}{25.4} + 5\right)$$

Ollerenshaw Index

Farmers are updated on fasciolosis risk as NADIS publish their predictions on their website; https://www.nadis.org.uk/parasite-forecast/. The UK fasciolosis risk for the September 2019 is presented in Figure 3.1. Predictions were based off rainfall and temperature data collected during August-October 2018 and May-June 2019.

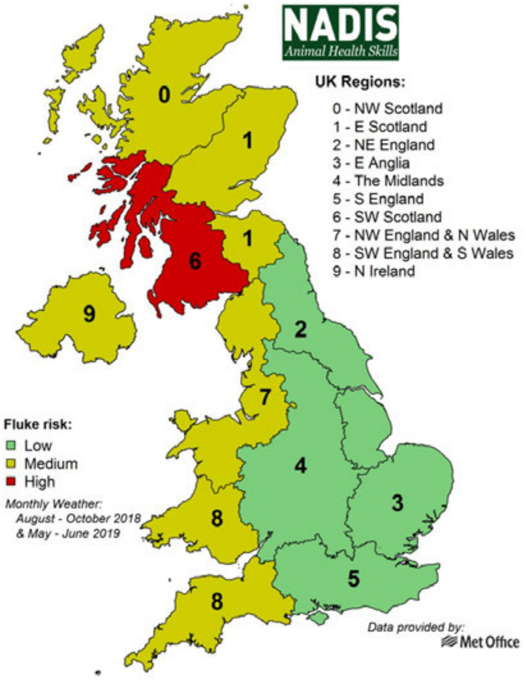

Figure 3.1 – The UK regional fluke forecast for September 2019 (NADIS, 2019)

The original purpose of the Ollerenshaw Index was as an early warning system for farmers, allowing time for the movement of livestock from potentially high risk pasture to somewhere of lower or no risk. Although this model is a good method for predicting disease on a national level, farmers are concerned about fluke risk at local level, specific to their farm and fields. For example, the risk posed by waders scrapes which are installed by the RSPB and qualify for European subsidy.

The prediction of local fluke risk is highlighted further when considering differences in farming environment even within the same region. Farms within the same post code region can experience different percentages of *F. hepatica* prevalence in their herds (Kuerpick et al., 2013). Other factors which have been shown to influence the risk of liver fluke include individual farm differences such as, herd size, pasture management, the length of the grazing season and anthelmintic use (Bennema et al., 2011). Regional liver fluke predictions cannot account for these individual differences in farming environment and management styles. Ultimately, liver fluke risk can only be determined by confirmation of viable, *F.*

hepatica metacercariae on pasture. The recovery and isolation of *F. hepatica* metacercariae from herbage or pasture is important for the determination of infective load and consequently the level of risk. There is very little published data on the quantity and distribution of metacercariae on pasture. This makes it difficult to discern how many and where metacercariae are present on a typical high risk field during a typical high risk year.

The recovery of intact liver fluke metacercariae from pasture samples would give an indication of the infective load on a grazing field at any time, irrespective of the environmental conditions. This type of detection method also allows for the absolute differentiation of metacercariae from the other life stages of this parasite which DNA detection does not currently allow.

Under some environmental stewardship schemes, farmers are encouraged to maintain and create wetland habitats for wetland birds and invertebrates (Scottish Government, 2016). These areas have the potential to overlap with habitat suitable for *G. truncatula.* Farmers have suggested that these areas may act as a possible risk factor for outbreaks of liver fluke, especially because there is a requirement to graze these areas (Pritchard et al., 2005). The advice often given to farmers for fluke control is to drain potential habitats of *G. truncatula,* if that is possible (Ollerenshaw & Rowlands, 1959). The use of molluscicides was previously advised, copper sulphate ($CuSO_{4)}$ and Frescon™ (N-tritylmorpholine) were highly successful at controlling *G. truncatula* and consequently reducing fasciolosis risk of farms (Crossland, 1976). Although effective, most of the chemicals used as molluscicides can damage other species within the same habitats and can even be toxic for livestock (Dreyfuss, Vignoles & Rondelaud, 2015). Molluscicide use on farms to reduce fasciolosis risk is now forbidden on environmental grounds. Consequently, there remains an imbalance between the best practice advice referring to the control of liver fluke for livestock and policies aimed at the conservation of wetland habitats to promote biodiversity.

The risk of liver fluke associated with grazing management, specifically in relation to the introduction of wader scrapes to improve wetland bird habitat has initiated collaboration between the Moredun Research Institute (MRI), the Royal Society for the Protection of Birds (RSBP), Scottish National Heritage (SNH), the James Hutton Institute and the Amphibian and Reptile Conservation Trust (ARC-Trust). The intention of wader scrape installation on farms is to promote biodiversity and create habitat for wading birds such as Lapwing (*Vanellus vanellus)* and Redshank (*Tringa tetanus)* (RSBP, 2017). Scrapes are indentations within the ground where water will be present for the majority of the year. Insects and other invertebrates populate these areas making them rich feeding areas for birds. Management of the scrape to retain good habitat for the wader birds requires some grazing by livestock. Low stocking density grazing on these areas keeps the sward length varied. This provides several different species of bird with their required sward length. Short, tussocks are good for the creation of nests and longer grass is ideal for concealing chicks during the breeding season (Durant et al., 2008).

Anecdotal reports from the above-mentioned organisations indicate reluctance from farmers to graze these areas due to a perceived risk of liver fluke to their stock. Farmers can receive money from the Scottish Government to install wader scrapes in fields on their farms (Scottish Government 2016). Wader scrapes will typically be filled with water, depending on the season (RSPB, 2010). The surrounding land is usually poor draining and suitable habitat for *G. truncatula.* Considering that outbreaks of fasciolosis are linked to the grazing of wet areas harbouring liver fluke-infected snails, there is a recognized risk of liver fluke to livestock who graze these areas. Additionally, the ingestion of wader scrape water infected with metacercariae by livestock is another perceived risk. There is evidence that cercariae are able to encyst on the surface of water by retaining small pockets of air. Boray (1963) observed cercariae encysting on the surface of water and it was assumed that some experimental mice included in this study were infected by drinking the floating metacercariae (Boray 1963). In the environment, metacercariae could float on the surface of water bodies until swallowed by a potential host while drinking.

The Netherlands Veterinary Services routinely use cellophane rafts for the collection of metacercariae from pastures to determine the level of infection risk to livestock (Gaasenbeek et al., 1992). This is particularly suitable for the Dutch system, as it takes advantage of the flooded nature of the grazing land. In a year of particularly acute cases of fasciolosis, 242 metacercariae were collected from one field over the course of 4 months (Gaasenbeek et al., 1992). Despite the success of this method it is not suitable for the pasture-based UK livestock systems where water bodies and wet areas are actively removed from pasture. The exception to this would be the inclusion or installation of water bodies to promote biodiversity on farms, for example, the installation of wader scrapes.

Typically livestock become infected with liver fluke after ingesting pasture containing viable metacercariae. The recovery of metacercariae from grass would be the most suitable method for understanding of liver fluke risk on farms in the UK. Despite the existence of a successful recovery method published by the Ministry of Agriculture Fisheries and Food (MAFF) in 1986 it is not used routinely because of Health and Safety concerns around the exposure of highly concentrated sulphuric acid to water. This method is also highly labour intensive, potentially requiring the collecting and processing of many kilograms of fresh grass material to determine metacercarial presence in just one areas of a field (MAFF, 1986).

A less hazard method of metacercarial recovery which could be applied to pasture is one developed by El-Sayad et al., 1997). They published a method for the removal of metacercariae from salad leaves in order to reduce the risk of human fasciolosis. Of the chemicals used, the authors found that 6% acetic acid, or commercial vinegar, was the most effective, capable of removing 100% of *F. hepatica* metacercariae on all plant species when metacercariae were exposed for 5 or 10 minutes (120ml/L). It is believed that the chemicals used reacted with the proteins of the cyst wall causing the bond formed between the plant surface and the cyst wall during encystment to break down, thus allowing the metacercariae to separate from the plant. According to the authors, vinegar did not cause any structural harm to the salad leaves and these could still be used for human consumption.

The El-Sayad et al., (1997) method for clearing metacercariae from salad leaves is significantly less hazardous than the MAFF (1986) recovery method. Vinegar could be applied in order to detach metacercariae from grass and a salad spinner can be used instead of sulphuric acid as a way to separate the grass from the metacercariae.

This chapter aims to evaluate the risk of fasciolosis to grazing livestock on wader scrapes over time. Field work, including the collection of snails and the deployment of cellophane rafts to recover metacercariae were used to do this. This chapter also evaluates and demonstrates the use of non-hazardous methods to recover metacercariae, encysted and re-adhered to both cellophane and grass.

Chapter 3: Detection of *F. hepatica* metacercariae on pasture/in the environment, with emphasis on the risk of fasciolosis associated with agri-environmental schemes

3.2 Materials and Methods

3.2.1 Field work at Kirkton and Auchtertyre farms

Kirkton and Auchtertyre farms, also known as the Hill and Mountain Research Centre for the Scottish Rural University College (SRUC), was the location for this field study. This is a 2200 ha Highland estate near Crianlarich in the West Highlands of Scotland. There were three sample sites; two newly installed RSBP wader scrapes (Figure 3.2a) and a drainage ditch. The drainage ditch was located within a separate 'fluky' field, known to have suitable habitat of *G. truncatula* after previous recovery of these snails from this site (Figure 3.2b).

It was thought that cellophane rafts (similar to one used by Gassenbeek, et al., (1992) could easily be transferred for use on the wader scrapes and the ditch sampled in this study, as they so similarly resemble flooded and ditched Dutch fields. Cellophane rafts were rudimentarily constructed out of 1.5 cm thick, 20 x 20 cm squares of polystyrene. Cellophane sheets (provided by Professor Neil Sargison) were attached to the polystyrene squares on both sides and fixed in place with two elastic bands. This was to account for the possible flipping over of the rafts caused by windy weather. The rafts were placed in all three of the sample locations and anchored down within grazing cages (kindly donated by the Centre for Hydrology and Ecology) to protect them from damage by either animals or the weather (Figure 3.2c).

Originally, sampling visits were intended to follow the frequency of Gassenbeek., et al (1992) which was to visit each site fortnightly, however, timings of visits were subject to the availability of people and the logistics of travel (Kirkton is ~75 miles from Edinburgh). At each site, two people would spend fifteen minutes searching for and collecting snails. To remain consistent, *G. truncatula* searching was restricted to the muddy edges of the scrapes and within the pool of the scrape. Other snail numbers were counted roughly; either recorded as around 20 (~20) or if too great to count within the time frame, counting stopped at 50 and the numbers were recorded as >50. If numbers were During each visit, cellophane sheets were collected from the rafts and new cellophane sheets deployed. If fresh faeces were observed near the scrapes those would be collected.

Upon return to the lab, snails were kept alive in Petri dishes, using fish feed as food. The snails would be checked intermittently for signs of cercarial shedding. Snails were subsequently frozen for further DNA testing.

3.2.2 Inspection of cellophane collected from deployed rafts

The day after collection the cellophane sheets were cut into small sections, as to fit the bottom of a Petri dish. Water was added, enough to cover the bottom of the dish and using a dissection microscope the cellophane pieces were inspected meticulously for encysted metacercariae.

Figure 3.2 Newly installed wader scrape at Kirkton and Auchtertyre farms (scrape 1) a). Ditch situated in 'fluky' field (b). Floating cellophane rafts within grazing cage at scrape 2 (c). *Galba truncatula in situ* at Kirkton and Auchtertyre scrapes (d) and (e).

3.2.3 DNA extraction – Snails

Gillian Mitchell carried out the following DNA extractions on snails collected from Kirkton and Auchtertyre. Using fine tweezers the bodies of snails were removed from their shells for DNA extraction. It was not possible, in all cases, to remove the body from the shell and so for these snails the shells were crushed in an Eppendorf using a micro pestle. Where needed 200µl of NF H_2O was added to aid crushing and to rinse the pestle. DNA extraction was then carried out using the DNeasy® Blood and tissue extraction kit (Qiagen, Germany). The protocol for DNA extraction was followed as per manufacturer's instructions except for two exceptions. Tissue was shaken in a water bath, with 180µl ATL buffer and 20µl proteinase K, at 56˚C overnight to completely lyse tissue. DNA was eluted in 100 µl of Buffer AE rather than 200 µl. Full description of protocol can be found in Appendix 1.

3.2.4 Polymerase chain reaction (PCR)

Gillian Mitchell carried out COX1 PCR amplification of *F. hepatica* DNA from snails collected from Kirkton and Auchtertyre. *F. hepatica* COX1 PCR was performed in 20 µl reaction volumes containing; 10x reaction buffer (Invitrogen, USA), 50mM $MgCl_2$ (Invitrogen, USA), dNTP mix (Invitrogen, USA), Nuclease free H_2O (Sigma) and 5 U Platinum *Taq* polymerase (Invitrogen, USA). The *F. hepatica* mitochondrial DNA fragment FhCox1 was amplified from metacercarial DNA using Cox 1 F 5' –GTTGGCATATTGCGGCTTAG-3' and Cox 1 R 5'-AGGGATCTGCACCTCAACTC-3' primers (Martínez-Pérez et al., 2012). Cycling conditions were as follows; 2 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 63°C for 30 seconds, extending at 72°C for 45 seconds and finally 10 minutes at 72°C (Biometra, Thermocycler gradient, Germany). PCR products analysed on a 2% agarose gel prepared using Tris-acetate EDTA (TAE) and stained using GelRed™ Nucleic acid stain (Biotium). Gels were run at 100v and visualized on AlphaImager™2200 (Alpha Innotech).

3.2.5 *F. hepatica* faecal egg count

Faecal egg counts (FRC) were performed by Gillian Mitchell using the Moredun FEC method. For each sample 3 g of faeces was mixed with 42 ml of water and poured through a metal strainer into a 250 ml beaker. The mixture was then poured through a 150 µl sieve into a 250 ml conical measure. The original beaker was half filled with water to resuspend any remaining eggs, the washing were then poured through the 150 µl sieve into the conical measure which was allowed to sit at a 20° angle for exactly 3 minutes. The supernatant was removed with a vacuum tube and one drop of 1% (w/v) methylene blue was added to the remaining sediment. Any eggs present in the stained sediment were identified under a light microscope (x16) using a marked petri dish (7cm diameter). Each sample dish was counted twice, the average number of eggs counted was then divided by 3 to give the number of eggs per gram (epg). An animal was considered positive with a burden of 1 or more epg.

3.2.6 Mechanical recovery of metacercariae from laboratory cellophane and grass

The following experiments were performed on cellophane and grass originating from laboratories and not the environment. These experiments were designed to be a proof of concept demonstration in the recovery and isolation of metacercariae.

The ideal study design called for the use of an *F. hepatica* infected *G. truncatula* colony which could be used to shed metacercariae on laboratory grown grass. However, in the absence of a snail colony, commercial metacercariae received encysted on cellophane were used instead. The recovery rate of metacercariae as received on cellophane was compared to the rate of metacercariae recovered after being allowed to re-adhere to both cellophane and grass.

The chosen method metacercarial recovery and isolation was based on one previously used to remove metacercariae from plants destined for human consumption(El-Sayad et al.,1997). This method is characteristically less hazardous than the MAFF (1986) method.

El-Sayad et al., (1997) explored the effect of running water and washing time on the removal of encysted *F. hepatica* on plants for human consumption. The use of different reagents to detach metacercariae from the plants without making them inedible was also explored. Vinegar (100 ml/L) was the most successful, removing 100% of metacercariae from leaves after 10 minutes exposure (El-Sayad et al., 1997). It was thought that the effect of washing and exposure to vinegar could be applied to removal and isolation of *F. hepatica* metacercariae from cellophane and grass. The use of a salad spinner was chosen over the use of running water as this method allows for conservation of water and allows for the convenient separation of grass from metacercariae suspended in water. It was thought that the centrifugal force of the salad spinner would release any encysted metacercariae from the cellophane and become suspended in water.

Recovery of *F. hepatica* metacercariae as received on cellophane

280 *F. hepatica* metacercariae (strain ‘Miskin’, Ridgeway Research, UK), encysted on one sheet of cellophane, was used in this experiment. This piece of cellophane was successively spun in a salad spinner for three, one minute intervals. Just water was used in the first treatment in order to gage of how many metacercariae were removed with just centrifugal force. The cellophane was then exposed to vinegar for 10 and then 20 minutes to before the second and third spinning intervals. The successive treatments are summarised below;

1. Cellophane placed into the inner part of a salad spinner with enough water to cover the bottom of the salad spinner. The cellophane was spun in the salad spinner for 1 minute and the water was decanted into a Petri dish for counting.
2. The same sheet of cellophane was placed in a Petri dish of vinegar (distilled, Tesco) for 10 minutes. Both the cellophane and vinegar solutions were spun for 1 minute. Any detached metacercariae were then decanted and counted.
3. Finally, the same cellophane sheet was placed in vinegar (distilled, Tesco) and left for 20 minutes. Vinegar and cellophane were then spun for 1 minute before counting the detached metacercariae under a microscope.

Recovery of re-adhered *F. hepatica* metacercariae from cellophane and grass

For the purposes of this experiment (and experiments of Chapter 5), grass (*Lolium perenne,* Homebase) was grown in the laboratory (Figure 3.3). This was to ensure confidence in the grass being completely free of metacercariae prior to the experiment. Grass was sown in compost and watered at regular intervals. After 1 month, the grass was harvested (Figure 3.3 c), cutting as close to the bottom of the stems without including soil in the samples.


SOWN 10.4.17
10.4.17 GRASS 'CYSTWORK'
GRACE CUTHILL
(a)

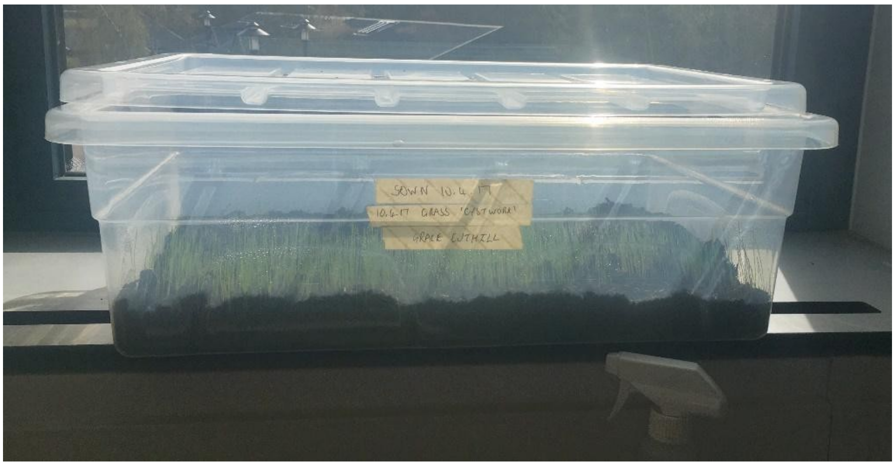

Figure 3.3 Laboratory-grown grass. (a) newly sewn grass in compost. (b) growth of grass after 1 week. (c) harvesting of grass.

Six laboratory-grown grass samples were used, each weighting 1 g. The grass samples were split into two groups, one group to be inoculated with 15 metacercariae each, the other

30 metacercariae each. These small sample numbers were chosen based off recovery rates referenced by Dreyfuss et al, (2005) from watercress. The numbers of metacercariae used to inoculate the grass and cellophane samples were chosen to best mimic numbers that might be present in environmental samples whilst also to allow for an understanding of method efficacy. Additionally, by having one set with 50% (15) of the other set (30) it was thought that some understanding as to the limit of the method could be gained. To explore differences in the rate of recovery or re-adhered metacercariae from grass compared to cellophane a concurrent group of six cellophane squares, measuring the diameter of a Petri dish, were prepared to give two groups, either containing 30 or 15 metacercariae each.

The *F. hepatica* metacercariae used for this experiment had originally been encysted on cellophane (Ridgeway Research, UK). Metacercariae were carefully dislodged from the cellophane they were supplied on with a cotton bud. The dislodged metacercariae were then transferred on to fresh grass or cellophane in a few drops of water and left undisturbed for 10 days.

To recover the re-adhered metacercariae 5 ml of vinegar (distilled, Tesco) was poured into the Petri dishes of grass and cellophane and left for 10 minutes. Each sample was processed separately. The grass or cellophane and vinegar solution was placed into the inner part of a salad spinner. With the lid attached the material was spun for 1 minute. Liquid was strained using a 100 µm sieve and recovered metacercariae counted.

3.2.7 Recovery of freshly encysted metacercariae from laboratory grass

Snails believed to be of the genus *Radix* were collected from wader scrapes at SRUC Hill and Mountain Centre. The exact species of the sails was not determined by DNA but by shell morphology (Gillian Mitchell). Snails were kept alive in Petri dishes of water, and fed a diet of dried fish food flakes. Every day the petri dishes were checked, observing the snails for cercarial shedding. Out of the many tens of *Radix sp.* snails collected many were observed to have metacercariae encysted on their shells and to be shedding metacercariae from their bodies. In the absence of an *F. hepatica* infected colony of *G. truncatula* it was thought that these actively shedding *Radix sp.* snails could be an adequate alternative to explore and demonstrate the recovery of metacercariae form grass in a controlled setting.

The metacercariae shedding from *Radix sp.* were morphologically determined to be *Notocotylus sp.* because of the morphological similarities between the metacercariae collected from scrape 1 and the description detailed by Besprozvannykh et al., (2013) and Assis et al., (2019). The three eyespots the proclivity to encyst upon the shell of the snail were two identifiable features of *Notocotylus sp.* This behaviour is thought to aid in the continuation of this parasite lifecycle as snails are a part of birds diet, wading birds included (Boyce, 2013). Water voles are also the definitive hosts of *Notocotylus* species (Boyce, 2013). Water voles had been observed at Kirkton and Auchtertyre farms by resident upland ecologist Dr John Holland (SRUC) and their presence was confirmed at the wader scrapes by the observation of rodent droppings around the edges of the scrapes.

5 snails were identified to be actively shedding metacercariae at the same time. These 5 snails were transferred onto 2 g of laboratory-grown grass in a Petri dish (Figure 3.4) and left for 2 days. Grass was inspected for the presence of encysted metacercariae under a dissection microscope. This grass was then placed into a solution of 120 ml of distilled vinegar (Tesco) and 880 ml of water and left for 10 minutes. 900 ml of the solution was strained using a 100 µm sieve. The remaining 100 ml of water/vinegar solution and grass was spun in a salad spinner for 1 minute and the mixture strained on top of the same sieve. Metacercariae were counted on top of the sieve using a dissection microscope.

Figure 3.4 – Laboratory-grown grass harvested and placed in Petri dish (a), 5 *Radix sp.* snails infected and shedding metacercariae on harvested grass

3.3 Results

3.3.1 Field work at Kirkton and Auchtertyre farms

At the beginning of this project, 2016, the wader scrapes were relatively new, having only been created a few months earlier. Prior to scrape installation these fields had not recently been grazed, it is not known if they were ever grazed. Apart from the stream that runs through the field of scrape one, the scrapes are the only water bodies on each site. It was noted that the numbers of invertebrates present in and around the immediate vicinity of the scrapes was very low. No plant life was growing in or on the edges of the scrapes. The field described as 'fluky', was a grazing pasture which included an area previously known to be a habitat for *G. truncatula* snails. This field was an established part of the grazing rotation and livestock were present there more often than in the fields containing the scrapes. This area displayed typical 'high fluke risk' characteristics; a ditch with slow running water, *Juncus sp.* reeds and poached areas of ground (Figure 3.b).

Over the three years of study, the sites of interest were visited a total of 15 times. Trips were made every two weeks subject to availability of people and logistics of travel. In total, 198 snails, either *G. truncatula* or *Radix sp.*, were collected over the 15 visits. As determined by PCR (Gillian Mitchell), 5 snails collected from the ditch were positive for *F. hepatica,* four collected on the first visit and one on 02/11/17.

During 2016, *Radix sp.* snails were highly abundant at scrape site 1. Snails were observed in numbers that were difficult to count within the sampling time frame. To compensate, counting stopped after 50 snails. No snails were observed at scrape 2 on any of the visits made during 2016. A single faecal sample from deer, collected from the edge of scrape 2, had an *F. hepatica* egg count of 1.67 epg. The other faecal samples from 2016 were from sheep and cattle. No eggs were found in any faecal samples taken from livestock during

2016. *G. truncatula* snails were only found within the ditch of the ‘fluky’ field and were only observed there in late September 2016.

In 2017, two more scrapes were installed into each of the fields containing scrapes 1 and 2. Both scrape 1 and 2 appeared more established as a habitat with the growth and appearance of more plants and animals in and around them. The ditch within the ‘fluky’ field was overgrown with rushes and grass and the mud level was much lower than in 2016.

Table 3.1 – Summary of field work carried out at Kirkton & Auchtertyre farms during 2016

Date	**Location**	**Snails**		**Samples collected**
		Radix	***Galba***	**Faeces (FH epg)**
18/08/16	Scrape 1	12 (7 alive)	0	X
	Scrape 2	0	0	X
	Fluke field	1	21 (7 dead)	X
01/09/16	Scrape 1	>50 (observed)	0	X
	Scrape 2	0	0	Deer (1.67)
	Fluke field	~20 (observed)	13 (9 dead), >50 empty shells	X
14/09/16	Scrape 1	>50(observed)	0	Sheep (0)
	Scrape 2	0	0	Sheep (0)
	Fluke field		11	X
30/09/16	Scrape 1	>50 (observed)	0	X
	Scrape 2	0	0	Cattle (0)
	Fluke field	~20 (observed)	22 (2 dead)	X
14/10/16	Scrape 1	>50 (observed)	0	X
	Scrape 2	0	0	X
	Fluke field	~20 (observed)	0	X
04/11/16	Scrape 1	>50 (observed)	0	X
	Scrape 2	0	0	X
	Fluke field	~20 (observed)	0	X

Radix sp.. snails were present on every visit to scrape 1 during 2017, and *G. truncatula* established a small population which disappeared from this site after July (dry). At scrape 2, the number of snails increased from 2016. *Radix sp.* snails were observed throughout the summer months and were not seen at this site after October. There were a number of *G. truncatula* snails sampled in August from scrape 2 but no live *G. truncatula* were found at this site during any of the other visits in 2017. In the ditch of the ‘fluky’ field, a small population of *Radix sp..* snails were observed but this population died out after August. The number of *G. truncatula* snails in the ditch of the ‘fluky’ field was completely diminished compared to 2016, with only one empty shell found at this site the whole year.

Table 3.2 – Summary of field work carried out at Kirkton & Auchtertyre farms during 2017

Date	**Location**	**Snails**		**Samples Collected**
		Radix	***Galba***	**Faeces (FH epg)**
16/06/17	Scrape 1	16	4	Deer (0)
	Scrape 2	0	2 empty	Deer (0)
	Fluke field	4	0	Sheep (0.33/0.66)
07/07/17	Scrape 1	22 (alive), ~90 dead	1 empty	Sheep (0); Rodent (0)
	Scrape 2	1	0	Deer (0)
	Fluke field	7 (+1 shell, 1 dead)	0	Sheep (0)
10/08/17	Scrape 1	3 alive, 18 dead	0	Sheep (0), Vole (0)
	Scrape 2	>50 (observed)	12 alive, 5 empty	Deer (0), Deer (0.1)
	Fluke field	2	0	X
02/11/17	Scrape 1	5 (attached to cellophane)	0	Sheep/Deer (0)
	Scrape 2	0	0	X
	Fluke field	0	1 (plus 1 empty)	X
23/11/17	Scrape 1	7	0	X
	Scrape 2	0	0	X
	Fluke field	0	0	X

Sampling in 2018 started in May which was much earlier than the previous years. The weather during June 2018 was uncharacteristically dry. No water was present in either scrape or the ditch during the first sampling visit of 2018.

Radix sp. snails were observed in numbers that were difficult to count within the sampling time frame within the mud of scrape 1 during 2018. *G. truncatula* was observed at most abundance at scrape 2 but live snails were not found after June. Only two *Radix sp.* snails and four *G. truncatula* snails were observed in the ditch of the ‘fluky’ field for the whole of 2018. *F. hepatica* eggs were found in three faecal samples taken from sheep grazing the ground around scrape 2 in September 2018

Table 3.3 – Summary of field work carried out at Kirkton & Auchtertyre farms during 2018

Date	**Location**	**Snails**		**Samples Collected**
		Radix	***Galba***	**Faeces (FH epg)**
28/05/18	Scrape 1	>50 (observed)	0	X
	Scrape 2	0	3	X
	Fluke field	0	0	X
02/06/18	Scrape 1	>50 (observed)	0	Deer (0)
	Scrape 2	0	6	X
	Fluke field	1	0	X
23/07/18	Scrape 1	8 (& 1 empty)	1 (2 empty)	10 x sheep (0)
	Scrape 2	0	8 (8 empty)	X
	Fluke field	1	4	X
24/09/18	Scrape 1	0	0	10 x sheep (0)
	Scrape 2	0	0	10 x sheep (9.33, 7, 1)
	Fluke field	0	0	X

Across the three years of field sampling, *F. hepatica* metacercariae were not observed on the cellophane deployed at any of the sites. Putative metacercariae were thought to be *Notocotylus sp.* because of the morphological details of three eyespots (Besprozvannykh et al., 2013 and Assis et al., 2019), were observed at scrape 1. Cercarial shedding of these metacercariae was witnessed in the lab from *Radix sp.* snails collected from scrape 1 (Figure 3.5 and 3.6). The metacercariae shed from *Radix sp.* would encyst upon the outside of the snail shell.

Figure 3.5 – *Radix sp.* snail collected from scrape 1. Metacercariae thought to be *Notocotylus sp.* encysted on shell (arrows)

Figure 3.6 – Putative metacercaria, thought to be *Notocotylus sp,* shed from *Radix sp.* snail in laboratory (8/11/16) (x40). Arrows indicate three eye spots, similar to the morphology of *Notocotylus sp*. described by Besprozvannykh et al., (2013) and Assis et al., (2019).

Five putative metacercariae were also observed on cellophane deployed in the ditch of the 'fluky' field, collected 7/7/2017 (Figure 3.7). Species identity of putative metacercariae could not be determined from the DNA extracted. *F. hepatica* COX 1 PCR determined that the DNA was not positive for the target.

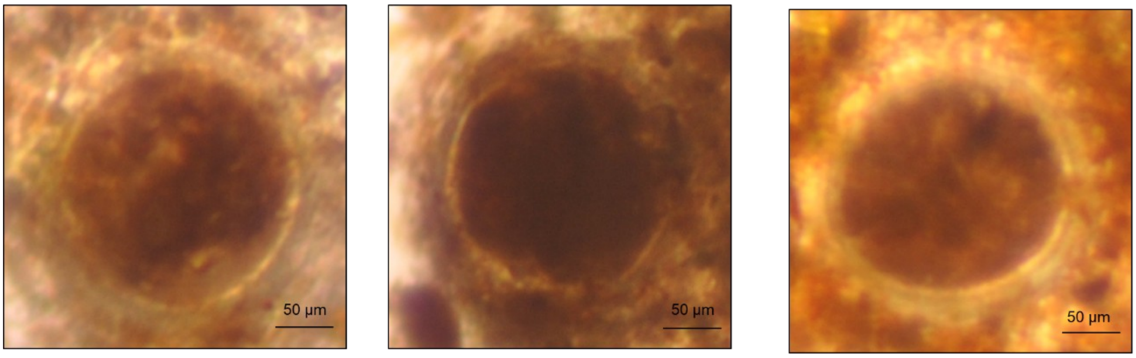

Figure 3.7. Putative encysted metacercariae found on cellophane deployed in the ditch of the ‘fluky’ field x40

3.3.2 Mechanical recovery of *F. hepatica* metacercariae from laboratory cellophane and grass with a salad spinner

The same single sheet of cellophane with ~280 encysted metacercariae underwent three successive treatments involving increased periods of exposure to vinegar and spinning in a salad spinner (Table 3.4).

Table 3.4 – Recovery of encysted metacercariae on cellophane

Treatment	Recovered metacercariae
1	40
2	21
3	63

Key;

1. 1 minute in salad spinner with water
2. 10 minutes exposure to distilled vinegar and 1 minute in salad spinner
3. 20 minutes exposure to distilled vinegar and 1 minute in salad spinner

A total of 124 of the original ~280 encysted metacercariae were recovered using the salad spinner and sequential exposure to vinegar. The last treatment recovered the most metacercariae out all three and the second treatment the least successful.

Re-adhered samples

Table 3.5 – Numbers of recovered, re-adhered *F. hepatica,* metacercariae from laboratory grass and cellophane

Overall metacercarial recovery was more successful from grass than from cellophane (Table 3.5). Total recovery of all metacercariae was not achieved. The numbers of re-adhered metacercariae recovered from grass ranged from 2-20 metacercariae. From cellophane the highest number of metacercariae recovered was 20, with 0 metacercariae recovered from one group.

Numbers of re-adhered metacercariae	**Numbers of recovered metacercariae**					
	Grass			**Cellophane**		
	a	b	c	a	b	c
30	2	8	20	2	9	20
15	11	5	11	6	0	3
Total recovered	57			40		

3.3.4 Recovery of freshly encysted metacercariae from grass

A total of seven *Notocotylus sp.* metacercariae were recovered from the grass exposed to infected *Radix sp.* snails using the salad spinner and vinegar technique. Figure 3.8 (a) shows an example of one of the encysted *Notocotylus sp.* metacercaria observed on the laboratory grass. Many *Notocotylus sp.* metacercariae were shed from the *Radix sp.* snails, the majority encysted on the side of the Petri dish (Figure 3.8 (b)).

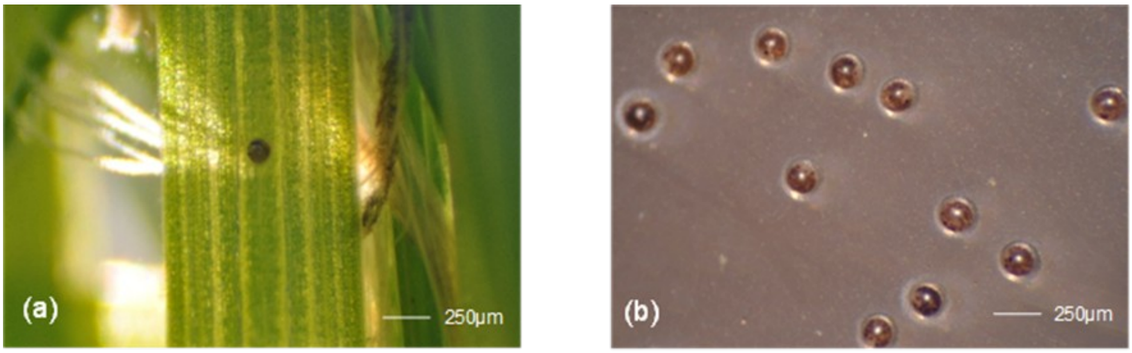

Figure 3.8– *Notocotylus sp.* metacercaria encysted upon grass (a), Newly encysted *Notocotylus sp.* metacercariae (b)

3.4 Discussion

The field work performed at the scrapes sites over a three year period has shown that wader scrapes can offer suitable habitat for *G. truncatula.* Snails positive by PCR for *F. hepatica* and FEC eggs counts from faeces found around the scrapes indicate that these areas can sustain the *F. hepatica* life cycle. Cellophane rafts were unsuccessful in recovering *F. hepatica* from the sample sites. Some *F. hepatica* metacercariae were recovered from cellophane and grass using the salad spinner and vinegar technique. Determining the efficacy of this technique was hindered by lack of resources.

3.4.1 Field work

The wader scrapes installed at Kirkton and Auchtertyre farms provided a rare opportunity to monitor liver fluke risk over time in a defined environment. Initially, both scrapes appeared very sparse with little vegetation around them or presence of invertebrate species in the water or on the muddy edges. The creation of wader scrapes is typically performed with mechanical diggers. Their excavation creates a lot of bare earth and newly-installed wader scrapes can appear quite barren to begin with. Despite the new scrapes' inability to support and provide habitat for many species early on, *Radix sp.* snails colonized scrape 1 quickly and in high numbers. The exact species of *Radix sp.* snail was unknown but the environmental tolerances of *Radix labiata*, for example, are described as wide-ranging (Strum, 2013). It has been suggested that the ability of *R. labiata* to tolerate extreme environmental conditions makes it a pioneer species in water bodies of mountainous areas (Strum, 2013).

The early appearance of *Radix sp.* snails and the large number observed throughout the study suggest that this species was already established on the farm land. Both scrapes were installed on marshy ground, unfavourable to grazing. The existing streams flowing through each field and used to fill each scrape were the original habitat for these snails.

Radix sp. and *G. truncatula* are of the same family, Lymnaeidae (Dreyfuss, Vignoles & Rondelaud, 2015). Being pond/mud snails, their habitats overlap. *Radix sp.* prefer to be fully submerged underwater. All of the *Radix sp.* snails observed and sampled, which were many, were found in the standing water of the wader scrapes. *G. truncatula* is amphibious, spending most of its time in the moist exposed mud around the edges of water bodies (Dreyfuss, Vignoles & Rondelaud, 2015). Although *Radix sp.* and *G. truncatula* snails can be found in the same habitats, the areas in which they prefer within these habitats are clearly defined. In France, *Radix balthica* was found in streams, whereas, *G. truncatula* was found on the outer edges of the channels created to drain water from the fields into the streams (Dreyfuss, Vignoles & Rondelaud, 2015). Moens (1991) also observed that *G. truncatula* was most likely to populate the periphery of ponds and streams. The muddy edges of wader scrapes are, theoretically, good habitats for *G. truncatula*. The installation of wader scrapes involves the disturbance and churning up of different soil layers. This fact aligns well with the observation that *G. truncatula* presence is highly associated with “perturbation”; defined as changes in soil sediments and deposits over time (Moens, 1981). The moisture content of the soil around the edge of a body of water will be high enough for the movement of snails without total submersion. All of the *G. truncatula* observed and sampled from the scrapes were found on the exposed mud on the edges of the scrapes and not in the water. This may explain why *F. hepatica* metacercariae were not found on any cellophane collected from the scrapes. The limited numbers of infected *G. truncatula* could also have reduced the possibility of the snail shedding cercariae in enough abundance to encyst on the cellophane. For this reason cellophane rafts may not be a suitable method for collecting metacercariae from these areas.

The cellophane rafts deployed in both scrapes and the ditch failed to collect *F. hepatica* metacercariae. 5 Putative metacercariae were collected from the ditch (07/07/2017) but there species identity could not be confirmed by DNA analysis. Other metacercariae thought to be *Notocotylus sp.* were found encysted on the cellophane deployed in scrape 1 and on the shells of many *Radix sp.* snails collected from the same scrape.

It is possible that *F. hepatica* metacercariae were being shed in the immediate area. There was positive *F. hepatica* COX1 amplification from *G. truncatula* collected on the 18/08/16 and on the 02/11/17. The absence of *F. hepatica* metacercariae from the cellophane deployed in the scrapes and ditch suggest that cercariae may not have been shed in enough abundance to have been observed encysted on the cellophane. Or cercariae did not reach the rafts because they were not shed into the water of either the ditch or the scrapes. Considering the numbers of *G. truncatula* collected over the 3 years and the low level of *F. hepatica* infection within these few collected snails this might explain why. This also brings into question the appropriateness of this method to collect *F. hepatica* metacercariae. As *G. truncatula* spends the majority of its time not submerged in water, it would be more appropriate to sample the vegetation surrounding the areas where the snail were found. Further work would incorporate metacercarial recovery methods from vegetation surrounding scrapes.

The poor practicality of using cellophane rafts to collected metacercariae also brings the suitability of this method into question. It was very difficult to find metacercariae on the cellophane when processing the collected sheets under a microscope. Sediment from the water made it almost impossible to see anything encysted on the cellophane. Further work could incorporate the use of the vinegar and salad spinner technique to recovery and isolate the metacercariae from collected cellophane. This would reduce processing time and would allow for more cellophane rafts could be deployed across each sample point thus also allowing for better coverage of the sampling area.

The barren, newly installed wader scrapes are not suitable habitats for *G. truncatula.* Pioneer species, including blue algae, green algae and plants are a food source for *G. truncatula* and though autotrophic succession would the first to colonise the barren, newly installed scrapes (Dreyfuss, Vignoles & Rondelaud, 2015). *G. truncatula* could not exist in these environments without a food source. There were trips during the sampling period when *G. truncatula* was not found but subsequent faecal egg counts of faeces sampled on the same day included *F. hepatica* eggs. This highlights how difficult it is to observe *G. truncatula* in the environment. Logistically it is challenging and it is also difficult to be systematic in sampling method. Much of the success in finding snails is subject to human competency and the climatic conditions of the day. Failure to find the snails on particular sampling trips does not equate to *G. truncatula* not being present in the environment. Repeat investigations would determine the populations of snails present on scrape sites before their installation. Field work would ideally be carried out at the same times each sampling day and only during the months with highest snail activity; June through till September (Hourdin et al., 2006). This would give a more consistent sampling method and perhaps a more comprehensive understanding of snail presence at scrape site

The potential for fluke risk on wader scrapes is also dependent on the fluke infection status of the stock that graze them. If the stock are fluke-free upon release onto the ground surrounding the scrapes, then there is relatively little risk of *F. hepatica* establishing in any *G. truncatula* colonies present. The scrape sites were grazed intermittently with sheep for a few days at a time during the course of the 3 sampling years. The fluke status of these sheep was unknown. Further work would include the integration of faecal eggs counts on the grazing sheep before and after they graze the wader scrapes.

The role of *Radix sp.* snails as an intermediate host for *F. hepatica* has been questioned and investigated. Using PCR, *F. hepatica* DNA was amplified from *Radix pelegra* sampled from upland sheep farms in Ireland (Relf et al., 2009). This finding explained outbreaks of fasciolosis in livestock grazing areas thought not to be suitable habitats for *G. truncatula.* Similarity, in Belgium and Luxembourg, 0.16% of *Radix sp.* snails (Caron et al., 2014) were confirmed by PCR to harbour *F. hepatica* larval stages. Shedding of *F. hepatica* cercariae by

Radix sp. was not confirmed in this study but had been previously witnessed from *R. labiata* in another (Caron, Lasri & Losson, 2007).

As is highlighted from the field work performed for this study and from the observations of Caron et al (2014), *Radix sp.* snails can occupy areas that *G. truncatula* cannot. The epidemiological consequences of this are that there may be more fluke risk areas and more areas with the potential to harbour risk. (Caron et al., 2014). The numbers of confirmed *Radix sp.* snails with *F. hepatica* infections are low and the risk transmission to livestock is relatively small. However, the knowledge that *F. hepatica* has a fast rate of genetic adaption and this has already been linked its adaptation to different habitats (Cwiklinski et al., 2015) this same potential could mean that *F. hepatica* will be able to effectively utilise other intermediate hosts.

Deer were already a permanent fixture of wildlife on the farm. It is presumed the deer would graze the grassland fields managed for sheep and cattle. Overlap of deer habitat and livestock grazing pastures was already in place. This overlap was allowing for constant re-infection of the pasture with *F. hepatica* eggs. The wader scrapes were perhaps an additional place for deer to get water.

The presence of liver fluke eggs within deer faeces collected over the last three years suggests that *F. hepatica* was successfully completing its life-cycle within the wild animal population visiting these sites, scrape 2, particularly. The location of scrape 2 was much more secluded and quieter than scrape 1 and the ‘fluky’ field. This perhaps allowed relatively shy deer to visit the scrape without being disturbed. Scrape 1 and the ‘fluky field’ were next to a farm track where farm traffic and walkers frequent every day making it less appealing for deer to graze. At scrape 2, deer could graze and utilise the habitat for long periods without being disturbed by humans or traffic. The levels of liver fluke infections in wild populations are unknown and are hardly ever investigated. Red deer (*Cervus elaphus)* and Roe deer *(Capreolus capreolus)* are often suspected as a reservoir species, acting as a constant source of reinfection of farmland pasture with *F. hepatica* eggs. Deer were observed in person at the scrape 2 site a number of times, while sampling. Their species identity was not identified but Red and Roe Deer are common across the Highlands of Scotland.

The prevalence of *F. hepatica* infections in Red *deer* was reported as high as 53%, in areas of the Scottish highlands (French et al., 2016). The low prevalence of liver fluke in Spanish populations of Roe deer questions their role as a reservoir host (Arias et al., 2013). Similarly to horses, roe deer may have some resistance to the development of fluke in the liver, meaning that the production of eggs by adults is greatly reduced (Arias et al., 2013). While there is contradictory evidence that Roe deer are more vulnerable to *F. hepatica* infections, with more severe cases of liver damage exhibited in Roe deer compared to Red de*er* (Munro, 1994). As research into *F. hepatica* infections in wild populations is rare the true effect of fasciolosis in deer species may never be fully understood. Hare *(Lepus europaeus)* and rabbits *(Oryctolagus cuniculus*) are other common reservoir hosts for *F. hepatica* on farms *(*Düwel, 1980 and Ziege, 2009).

Wader scrapes have the potential to be areas of fasciolosis risk to livestock, however, the level of risk to livestock depends on a number of factors. The age of the scrape is one factor. A newly-installed scrape would not necessarily be a high risk area for *F. hepatica* if the surrounding area is not already a habitat for *G. truncatula.* In the newly installed wader scrapes *G. truncatula* was not observed until roughly a year after the start of visiting and sampling these areas. *G. truncatula* could have been present in these areas but these areas were not canvased prior to wader scrape installation. A possible route of introduction for *G. truncatula* to scrape 1 is through the stream that connects to ‘fluky’ ditch and is upstream of scrape 1. Both scrape sites could have had *G. truncatula* introduced by the wading birds visiting these sites. The dispersal of aquatic organisms, including *G. truncatula*, by wetland birds has been implicated in the spread of this gastropod around the world. It has been suggested that by incomplete digestion or by attachment to the body of birds, *G. truncatula* has been dispersed to new areas (van Leeuwen, 2012). This potentially has led to the subsequent spread of *F. hepatica* to new areas.

3.4.2 Recovery of metacercariae from laboratory grass and cellophane

The pilot use of vinegar and a salad spinner to recover and collect metacercariae from cellophane and grass was inspired by the paper published by El-Sayad et al., (1997). The use of vinegar and a salad spinner, in this project, to recover encysted *F. hepatica* from cellophane was minorly successful. It appears that 20 minutes exposure to vinegar releases more metacercariae than 10 minutes, 60 vs 21. However, there are limitations to the experimental design and statistical analysis was not performed which makes it difficult to draw any real conclusions. The fact that the same cellophane sheet was used successively for each treatment doesn't allow for any understanding of recovery rates. Access to encysted *F. hepatica* metacercariae was limited to sourcing metacercariae commercially, which was expensive. The experiment was designed to make best use of the encysted *F. hepatica* metacercariae that were available whilst also keeping costs down. Future work would include repetitions of each treatment performed on single groups of metacercariae. Experiments would be designed to investigate longer intervals of vinegar exposure, longer washing times and higher centrifugal force on the recovery rates of encysted metacercariae. Statistical analysis would be incorporated to assess the efficacy of this method.

Re-adhered metacercariae were used in place of freshly shed metacercariae. By spiking grass and cellophane with 15 or 30 metacercariae the experiment hoped to allow for the best emulation of metacercarial numbers that might be recovered from environmental samples. The recovery rates from both cellophane and grass, regardless of the number of metacercariae present, was variable. This study design has an advantage over using a shedding snail colony as the number of metacercariae present in the samples is known. However, re-adhered metacercariae may not have the same strength of bond created between metacercarial wall and the surface on which the metacercariae freshly encyst. More metacercariae were recovered from grass than cellophane. Metacercariae may have re-adhered more readily to the cellophane as this has a smoother surface than blades of grass. To obtain reliable recovery rates experiments should utilise freshly encysted metacercariae on grass.

The use of vinegar and salad spinner to recover *Notocotylus sp.* metacercariae from grass was successful. If repeated the success of the method would be tested on grass infested with *F. hepatica* metacercariae. An infected *G. truncatula* colony would be used to shed metacercariae on the grass. This grass, which would emulate real pasture samples, can then be used in metacercarial recovery and isolation experiments. Quantification of metacercariae on grass before recovery methods are performed would be helpful in determining the efficacy of the recovery. Currently, the only way to count metacercariae on vegetation is with light microscopy. Vegetation can be inspected under a microscope for the presence of encysted metacercariae, however, this is very challenging time-consuming and does not always yield a high number of identified metacercariae for the amount of fresh grass that needs to be processed. A study of metacercarial load on 247 beds of watercress, in France, resulted in a mean of 0.9 metacercariae per bed of watercress over a 9 year period (Dreyfuss et al., 2005). Similarly low numbers of metacercariae were recorded on watercress beds in a more recent study, performed over 14 years. In 1999, the year with the highest count, a mean of 4.6 metacercariae were counted over 32 beds of watercress (Dreyfuss et al., 2005).

Correct identification of metacercarial loads using microscopy is challenging when considering the volume of herbage that needs to be inspected and the fact that metacercariae appear almost translucent on grass (Figure 3.9).

Figure 3.9 – Spiked, laboratory-grown grass with *F. hepatica* metacercariae

This is made all the more challenging when considering that samples of vegetation or pasture could include many different forms of detritus which could impair the visual quality of the sample.

Metacercarial recovery and isolation methods remove the challenges of metacercarial identification by light microscopy. The use of vinegar and salad spinner was successful in recovery and isolation metacercariae from grass grown in the lab. However, translation of this to use with grass samples collected from farms would also incur the same problems as light microscopy, with sample size and detritus hindering the process. Centrifugation of pasture samples containing metacercariae and step solutions of density gradient media e.g. Percol® and Metrizamide® was successful in separating metacercariae from plant material (Fry, et al., 1984, 1985). Not only did this allow for contamination-free samples of metacercariae but this method also allowed for the separation of viable and non-viable metacercariae into different fractions allowing for the use of metacercariae in further experiments (Fry et al., 1984, 1985).

Future work could incorporate the Fry et al., (1984, 1985) step to the vinegar and salad spinner method demonstrated in this project. Large, detritus filled samples onto which *F. hepatica* metacercariae have been shed could processed with varying degrees of exposure time to vinegar and spinning time in a salad spinner. The recovered solutions could then undergo centrifugation with density gradient media to separate detritus from metacercariae. Additionally, the viability of the recovered metacercariae in both the ‘viable ‘and ‘non-viable’ factions could be checked with an excystment assay, thus verifying that the method can separate viable and non-viable metacercariae.

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3.5 Conclusion

From the field work carried out on the same wader scrapes, over three years, it was observed that *G. truncatula* could utilise these areas as habitats. DNA amplification and FECs confirmed the presence of *F. hepatica* within the populations of *G. truncatula* and sheep present on the farm, highlighting that there is a risk of fasciolosis to livestock grazing wader scrapes. The level of fasciolosis risk from wader scrapes installed on farms is dependent upon individual situations and farmers must assess the risk according to the current status of their farm. Detailed and consistent sampling of snails these areas are needed to determine risk based on the presence of *G. truncatula* habitat. Performing consistent sampling during the time of highest snail activity will improve sampling success. It is also important to gain an understanding of the historic presence of *G. truncatula* on the farm especially in the area where scrapes are to be installed. Cellophane rafts may only be effective in the collection of metacercariae if the population of *G. truncatula* is large and the infection rate high. The recovery of metacercariae by cellophane rafts could be improved by increasing the number of deployed rafts across the sampling area. Isolation of the metacercariae from the collected cellophane can be very difficult. This step could be improved with the incorporation of a washing and straining method similar to the recovery of metacercariae from grass.

In order to attain balance between two competing objectives, fluke control and biodiversity of farms, there will need to be improved engagement between environmental policy teams, land managers and livestock farmers.

The recovery of metacercariae from grass and cellophane using vinegar and a salad spinner was demonstrated but the results were poor. The use of previously encysted metacercariae to purposefully spike grass and cellophane was advantageous for the knowledge of how many cysts were present in the samples but this unfortunately meant that the samples were not representative of real contaminated pasture samples. In the absence of a *G. truncatula* colony, the use of *Radix sp.* shedding *Notocotylus sp.* cercariae was a useful alternative to create representative contaminated grass samples. The use of vinegar and a salad spinner was successful in recovering *Notocotylus sp.* metacercariae from the grass however, without knowledge of how many metacercariae were encysted the effectiveness of the method was unknown. Future development of this method of metacercarial recovery should utilise *F. hepatica* shedding *G. truncatula*. This situation would be more conducive to the application of these method to real pasture samples as *F. hepatica* metacercariae could be purposefully shed on to grass. Following the determination of method efficacy this method could be further enhanced to be applicable to grass samples taken from farms.

Chapter 4: Survival of *F. hepatica* metacercariae within silage and at pH associated with silage fermentation

4.1 Introduction

The risk of fasciolosis is based on the presence of viable *F. hepatica* metacercariae on pasture. Grazing livestock can become infected when they ingest grass or other forage contaminated with viable metacercariae. Across most of the United Kingdom (UK) and Europe livestock will be housed during the winter. Some stock, for example intensive dairy herds, can be housed all year-round. In the UK grazing grass is not a viable option to maintain animal condition for either winter housed or permanently housed stock, farmers can choose to feed a diet including preserved forages, such as grass silage or hay during the winter. Grass silage is made by placing cut grass in pits/clamps or bales, during the summer and creating the best conditions (low oxygen) for its fermentation by the microbes naturally present on the grass.

Preserved forages offer a low cost, effective feeding option during a time where grass growth is poor and housing costs are high. Anecdotal reports suggest that some cattle that have been housed all year round and have not had any access to outdoor grazing, have become infected with liver fluke. It is not apparent in these cases where the animals might have come into contact with an area contaminated with metacercariae. A common link between these reports is the feeding of grass silage to the livestock and farmers believe this to be the source of infection.

Historically, farmers have expressed concerns about the longevity of viable metacercariae in silage and the risk posed to their livestock (Dawes, 1965; Kendall, 1965). In support of these unproven suspicions is a recent published report of the appearance of fasciolosis in un-grazed Danish dairy herds. It was argued that one possible source of infection could have been the feeding of grass and hay contaminated with metacercariae (Takeuchi-Storm, et al 2017). There exists very little literature describing the effect of ensiling on the viability and infectivity of liver fluke metacercariae. Influencing factors of metacercarial survival when ensiled may include; the method of ensiling, the type of crop and the amount of time spent in the ensiling conditions (John et al*.,* 2019). The presence of metacercariae on freshly cut grass for silage is very likely if there are infected *G. truncatula* snails in the same habitat. Sufficient moisture and temperatures about 10˚C are needed for the survival of snails and for the shedding of metacercariae onto pasture (Kendall, 1965). Indeed, there is a possibility of habitat suitable for *G. truncatula* to be present in silage or hay fields (Knubben-Schweizer & Torgerson, 2015).

It is assumed that the likelihood of viable metacercariae being present in silage is low, although it is accepted that the fermentation conditions of silage vary greatly, and depend on many factors. All the techniques for making and preserving grass silage aim to allow for fermentation under the influence of lactic acid bacteria (LAB) and anaerobic conditions. The challenge for farmers is to do this and avoid nutritional losses occurring through poor fermentation, such as reduced dry matter (DM) (Borreani et al., 2018). Key to the success of this process is the creation of anaerobic conditions and achieving low pH to prevent and reduce the activity of spoilage microorganisms.

There is some relatively old/early evidence that metacercariae can survive the fermentation of grass silage that was harvested in wet conditions. Fasciolosis has been reported in rabbits fed hay, contaminated with *F. hepatica* metacercariae, which had been previously harvested in wet weather and stored for 8 months (Marek, 1927, cited by Olsen, 1947). It could be argued that the hay described and fed to the rabbits in this study was actually a haylage because the grass was preserved wet rather than dry. Haylage is a type of grass silage, usually haylage will have higher DM than conventional grass silage.

pH is an important factor in grass silage fermentation. To prevent the growth of harmful, unwanted bacteria, such as *Listeria* (which causes Listeriosis), a pH of 4.2 or below is required (Perry & Donnelly, 1990). A decrease in silage acidity through soil contamination or exposure to oxygen could result in poor fermentation. Very little is known about the effect of pH on metacercarial viability. However, Tarczynski & Podkowka (1964) did suggest that lower pH negatively affects metacercarial viability. Fasciola infection was not found in rabbit hosts after they consumed silage contaminated with *F. hepatica* metacercariae. They suggested that metacercariae lose their infectivity as early as the first day of exposure to the fermentation process. The production of lactic acid and the subsequent drop in pH was explained as the main reason for the loss of infectivity in metacercariae (Tarczynski & Podkowka, 1964).

The aim of this Chapter was to determine the longevity of viable *F. hepatica* metacercariae at different pH associated with silage fermentation, and to determine the longevity of *F. hepatica* metacercariae in silages of different qualities, produced under laboratory conditions.

4.2 Materials and Methods

4.2.1 Survival of *F. hepatica* metacercariae in 10% lactic acid solution; pH4, 5, and 6

Experimental set-up

An investigation into the survival of metacercariae at different pH was motivated by the suggestion of Tarczynski and Podkowka (1964) that low pH was the reason behind loss of metacercarial viability during ensilage. Lactic acid is the dominant acid produced during this process. Generally, the lower the pH the better the quality of the silage so pH 4, 5 and 6 were chosen to represent typical silage pH.

Approximately 400 *F. hepatica* metacercariae (strain ‘MISKIN’, shed ~15/4/17, Ridgeway Research, UK), were used in this experiment, which was conducted over 4 weeks;.10 % lactic acid solutions were prepared by gradually dissolving lactic acid (Thermo Scientific) in 1M HEPES (Sigma), until pH4, pH5 and pH 6 were achieved. Approximately 100 metacercariae were placed in 1ml of each lactic acid solution and 100 metacercariae were placed in sterile water to act as a control group. Both the control and experimental groups were incubated at 4 ºC, in a fridge, to keep temperature conditions controlled. At 4 weeks the metacercariae were recovered and subjected to an excystment assay to determine metacercarial viability. 4 weeks was chosen as this is the minimum period grass can be ensiled before feeding to livestock, as advised by Dave Davies (Silage Solutions)

Excystment assays

The excystment assay used was developed from the work carried out in Chapter 2. This assay was based on that published by Hernández-González et al., (2010). From the experiments performed in Chapter 2 Minor changes were made to the methods to accommodate the equipment and resources available.

Assays were performed *in vitro* at the same time in four separate batches. Activation media consisted of 1 ml of 0.2M sodium dithionite (Thermo Scientific) dissolved in 10ml of cold carbonated, distilled water (Highland Spring). This solution was incubated at 39ºC for ~15 minutes until a cloudy precipitate was formed. Activation of the metacercariae was achieved by incubating metacercariae in the activating media for 1 hour at 39ºC. Before the addition of emergence media, metacercariae were washed twice with warm distilled water. Metacercariae were then transferred into this medium containing 10% (v/v) sheep bile (Dryden Farm), in 5ml of Hanks' balanced salt solution, and 1 ml of 30mM HEPES (Thermo Scientific). Metacercariae were incubated at 39ºC in the emergence media for a total of 5 hours, with the collection of juvenile fluke beginning after 2 hours.

Viable metacercariae were determined by the emergence of motile juvenile fluke as viewed under a dissection microscope (Figure 4.1). Non-viable metacercariae were identified that did not excyst after 5 hours in the emergence media.

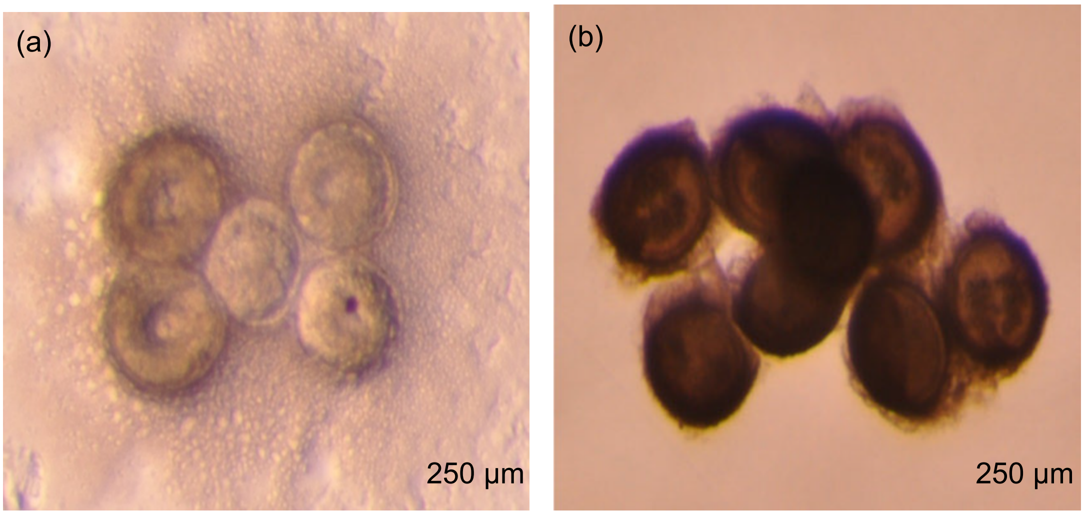
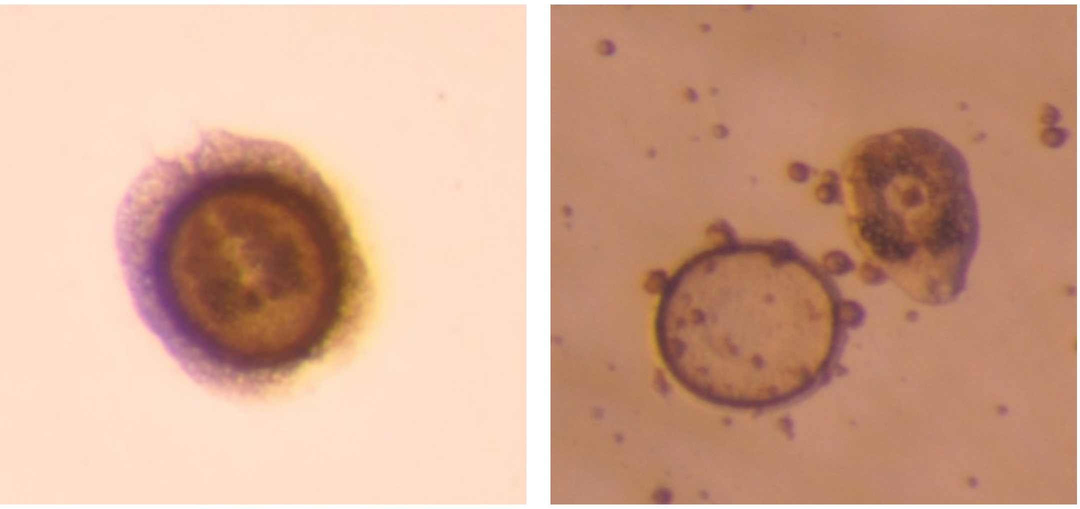


Figure 4.1 - *F. hepatica* metacercariae - Desiccated metacercariae (a), aggregate of viable encysted metacercariae (b), viable metacercaria prior to excystment (c) and newly excysted juvenile with empty cyst wall (d).



4.2.2 Survival of *F. hepatica* metacercariae in 10% lactic acid solution (pH4) an *in vitro* time-course 4-16 weeks

Experimental set-up

The premise for the experiment was to improve our understanding of the survival/viability of metacercariae over time at the optimal pH for silage fermentation i.e. pH 4.

The results of the previous experiment showed that the viability of the metacercariae was high at pH 4, 5 and 6, after 4 weeks incubation. Based on these results, an experiment with double the amount of metacercariae and longer exposure time was designed to gain a more detailed understanding of metacercarial survival at the ideal pH for silage fermentation, 4. 800 *F. hepatica* metacercariae were used in this experiment, conducted over 16 weeks; strain 'MISKIN', shed ~10/4/18 (Ridgeway Research).

A 10 % lactic acid solution was prepared by gradually dissolving lactic acid (Thermo Scientific) in 1M HEPES (Sigma), until pH4 was achieved. 4 groups of 100 metacercariae were placed in 1 ml of lactic acid solution (pH4) each. 4 equivalent control groups of 100 metacercariae were placed in 1ml sterile distilled water. Both the control and experimental groups of metacercariae were incubated at 4 ºC under laboratory conditions. At 4, 8, 12 and

16 weeks, a group of metacercariae and the equivalent control groups were excysted, as below, to determine metacercarial viability. The frequency of excystment was determined to best understand the rate of decline in metacercarial viability over the 16 weeks.

Excystment assays

Assays were performed *in vitro* at the same time in two separate batches. Assay protocol was the same as the previous section (6.2.1).

4.2.3 Silage fermentation

Silage fermentation is a multi-factored process. It was important to gain some understanding of metacercarial survival under, or as close as possible to, genuine grass ensilage conditions. Previous experiments explored only one factor of silage fermentation, pH. To gain a representative understanding of metacercarial survival under ensilage conditions it was thought that these should best be replicated, best as possible, in a laboratory setting.

Three samples of grass silage were collected from farms in Wales. The silages were categorised as ‘high’, ‘medium’ and ‘low’ quality. This was determined subjectively by observation of the smell and colour of the silage by an acknowledged expert. The subjective judgement was based on years of personal experience gained by Dave Davies (Silage Solutions Ltd, UK) in the field of grass silage fermentation. Immediately after sampling, the silages were sealed using in a vacuum pack in order to retain their quality during transport.

4.2.4 Silage quality analysis

To determine fermentation quality and other silage characteristics, near-infrared spectrophotometry (NIR) was performed on two sub-samples of each sample type. The quality of each silage sample was determined at week 16 of the experiment. This method was performed by the Analytical Services Department of the Scottish Agricultural College (Edinburgh). Appendix 5 for full reports.

4.2.5 Sub-sampling and spiking silage

1600 *F. hepatica* metacercariae, strain ‘Italian’, shed ~12/4/18 (Ridgeway Research). The numbers of metacercariae used was decided based on the fact that a reliable recovery method was not available and metacercariae maybe lost during the process. Numbers of metacercariae were also limited to timings and cost. Shedding date and delivery had to align with the delivery of silage. Thus the experiment could be set up in a consistent manner on the same day.

Time intervals of 4, 8, 12 and 16 weeks were designated for the recovery of metacercariae from the ‘high’ quality silage sample. Recovery of metacercariae from the ‘medium’ and ‘low’ silages were designated one time point of 14 weeks after spiking with fluke cysts. This was because the number of metacercariae purchased for this project was not sufficient to allow for a similar range of timepoints using ‘medium’ and ‘low’ silages. A summary of the experimental set-up is outlined in Table 4.1.

The ‘high’ quality silage was split into four groups of three 150g sub-samples. ‘Medium’ and ‘low’ silages were spilt into two groups of two 150g subsamples each. Cost and logistics of obtaining metacercariae limited the experiment to two groups for the ‘medium’ and ‘low’ silages

Using a 10µl pipette, each bag of silage was spiked with 50 metacercariae before being re-sealed within the vacuum pack. The sealed silage bags were stored in a dormant incubator at room temperature (~21˚C) throughout the experiment. The incubator was switched off but kept the silage bags intact and safe from potential puncture.

Control groups of 50 metacercariae were set up simultaneously to the experimental silage groups. Control metacercariae were placed in 1.5 ml Eppendorf tubes with sterile water and stored at room temperature (~21˚C).

Table 4.1 – Silage fermentation; Experimental design

Silage Type	Number of sub-samples per week								Total number of cysts present at the start of the experiment
Weeks	4		8		12		16		
	Exp	Con	Exp	Con	Exp	Con	Exp	Con	
High	3	3	3	3	3	3	3	3	1200
Medium	-	-	-	-	2	2	-	-	200
Low	-	-	-	-	2	2	-	-	200

4.2.6 Recovery of metacercariae

Each bag of silage was processed separately. The method of metacercarial recovery used was based on one published by (O’Shaughnessy et al., 2018). Metacercariae were recovered from the silage by jet-washing the silage over combined 300 µm and 100 µm metal sieves. Any material retained on the 100 µm sieve was backwashed into petri dishes and metacercariae were observed and counted under a dissection microscope.

Metacercarial viability was determined using the Hernández-González et al., (2010) excystment protocol with minor changes, as previously discussed in section 2.3.3.

4.2.7 Statistical methods

Minitab® 17 statistical software was used to perform Chi-square test for association. Chi-square was performed on the experiments investigating the effect of 10% lactic acid at pH 4, 5 and 6 on metacercarial viability over 4 weeks and the effect of 10% lactic acid at pH 4 overtime, a time course of 4-16 weeks.

4.3 Results

4.3.1 Survival of *F. hepatica* metacercariae in 10% lactic acid solution; pH4, 5, and 6

The viability of metacercariae was not significantly affected by the pH of the lactic acid solutions over 4 weeks (Table 4.2).

Table 4.2 – Viability % of metacercariae after 4 weeks at pH4, pH5 and pH6

	pH4	pH5	pH6	Control
Metacercarial Viability %	88	82	91	82

After 4 weeks incubation in lactic acid solutions the viability of the metacercariae, as determined by excystment assays, was not significantly different from that of the control group.

4.3.2 Survival of *F. hepatica* metacercariae in 10% lactic acid solution; pH4 an *in vitro* time-course 4-16 weeks

Viability of both the experimental and control metacercariae remained similar until 12 weeks, after which time the viability in the pH4 sample gradually decreased (Table 4.3). A statistically significant difference in viability was observed at 16 weeks; 38% viability in the experimental group versus 72% in the control group ($p<0.05$).

Table 4.3 – Percentages (%) of viable metacercariae in lactic acid solution (pH4) and water

Weeks	Control	pH4
4	91	97
8	65	67
12	71	85
16	72	38

Chi-Square – P-value =0.008

4.3.3 Silage fermentation

The number of metacercariae recovered from the different silage samples was relatively poor and varied from week to week and also between the silages (Table 4.4). ‘Recovered’ metacercariae refers to the number of metacercariae collected during the recovery process from silage. ‘Viability’ describes the number of recovered metacercariae that were able to excyst. Recovery rates ranged from 2 to 74 metacercariae out of 150 originally used to spike the silages.

Table 4.4 – Total number of metacercariae recovered from ‘high’, ‘medium’ and ‘low’ quality silages, their viability and the viability of equivalent controls incubated in water at 21°C

Weeks	‘High’ quality silage (150 metacercariae per week)		Water @ 21°C (150 metacercariae per week)
	Recovered	**Viable**	**Viable**
4	24	14	75
8	38	0	55
12	2	0	42
16	74	0	7
12	**‘Medium’ quality silage (50 metacercariae per sample)**		**Water @ 21° (50 metacercariae per sample)**
	Recovered	**Viable**	**Viable**
	30	0	6
12	**‘Low’ quality silage (50 metacercariae per sample)**		**Water @ 21°C (50 metacercariae per sample)**
	Recovered	**Viable**	**Viable**
	2	0	9

Viable metacercariae, as determined by excystment assay, were only observed from the group recovered from ‘high’ quality silage after 4 weeks incubation. A small number of the recovered metacercariae remained viable in silage for 4 weeks – 14 out of 24 (Table 4.4). 75 (out of 150) metacercariae from the relevant/equivalent control group, incubated at 21°C in water, were viable at week 4. Viability of the control groups steadily declined from weeks 8 and 12 but dropped dramatically to 7 out of 150 at 16 weeks.

Metacercarial recovery from the ‘medium’ quality silage was higher than from the ‘low’ quality silage. No viable metacercariae were observed from either of the groups. The viability of the complementary controls groups in water at 12 weeks were considerably lower than that of the complementary control group for ‘high’ quality silage at 12 weeks – 6 and 9 versus 42 viable metacercariae.

4.3.4 Silage quality analysis

The subjective assessments of silage quality are reflected in the results of NIR. With the ‘high’ quality silage having the most optimal levels of all the variables and the ‘low’ quality with the least optimal levels in all variables (Table 4.5). Each silage type was assessed twice, using two sub-samples, labelled 1 and 2, respectively. ‘high’ quality silage was observed/found to have just below ‘good’ pH and high lactic acid content, while the Volatile Fatty Acids (VFAs) levels were considered to be ‘average’. The pH of ‘medium’ silage was also considered to be ‘good’ but the lactic acid content was described as ‘low’ and the VFAs ‘very poor’. The pH of the ‘low’ silage was ‘average’, the lactic acid content ‘low’ and the VFAs levels were ‘very poor’.

Dry matter (DM) content was very similar between each sub-sample for each silage quality type. The DM was highest in the ‘high’ quality silage samples and lowest in ‘low’ quality silage samples. There was a slight difference in DM content of the ‘medium’ and ‘low’ quality silages, with the ‘medium’ silages only just having the higher DM.

A full report from SAC ASD can be found in Appendix 5

Table 4.5 – NIR results for ‘high’, ‘medium’ and ‘low’ quality silages

Silage variables	‘High’	‘Medium’	‘Low’
	Sub-samples		

	1	**2**	**1**	**2**	**1**	**2**
pH	4.1	4.1	4.7	4.6	5.2	5
Lactic acid (g/kg)	109.8	106.1	21.7	26.1	15.7	30.2
VFAs (g/kg DM)	44	42.7	75.7	80.7	61.6	60.5
Dry Matter (g/Kg)	315	328	281	261	255	268

4.4 Discussion

This Chapter aimed to determine how long *F. hepatica* metacercariae remain viable at different pH associated with the silage fermentation process, and to determine the longevity of *F. hepatica* metacercariae in silages of different qualities, performed under laboratory conditions.

From the experiments performed it was concluded that metacercariae can survive at least 4 weeks in lactic acid solution (10%, 4°C) at pH 4, 5 and 6. The levels of viability were high in all groups i.e. 82% excystment or higher. No significant difference in metacercarial viability was observed between groups or compared to the control metacercariae incubated in water ($p > 0.05$).

The length of metacercarial survival at optimal pH of silage fermentation was also investigated. It was shown that 38% of metacercariae can remain viable for up to 16 weeks in pH 4 lactic acid solution (10%, 4°C). This is significant when compared to the viability of the group at 12 weeks (85%). As the decline in viability was gradual and similar across all groups, the dramatic decrease in viability at 16 weeks in lactic acid could be the result of the effect of pH 4 over time. This was not simply a feature of time in incubation, because the control group, maintained in water, retained good viability throughout e.g. 72% at week 16. pH alone doesn't appear to be the main determinant of cyst viability, only 9.33% or 14 metacercariae ensiled in 'high' quality silage, with a pH of 4, were able to excyst after 4 weeks.

Theoretically, it is possible that the lactic acid solution degraded the cyst walls of the metacercariae. Prolonged exposure to lactic acid could have slowly degraded the cysts walls and could have accelerated the decline in metacercarial viability. Degradation of the outer wall renders the inner cyst, containing the encysted fluke, vulnerable to damage. *F. hepatica* metacercariae have a complex, 4 sub-layer, outer cyst wall designed to keep the inner cyst protected against environmental factors, including any compounds that may be poisonous or degrading (Dixon, 1965). However, countering this theory is the observation that metacercariae are capable of surviving and excysting in the pH conditions within the digestive system of livestock. As metacercariae pass through the stomachs of ruminants they will encounter pH's ranging from 2-6 (Bergmann, 2017).

It is generally accepted that the lower the pH, the more stable the silage fermentation and the better the preservation of the silage. Silages of good quality will typically fall into the pH range of 3.7-4.7 and pH is commonly used as a rudimentary test of silage quality. The pH of silages is the result of a combination of acids, of which lactic is the most abundant. Acetic, butyric and propionic acids are also involved in silage fermentation. The percentage of each of these acids is linked to silage quality, with higher proportions of acetic, butyric and propionic acids present in lower quality silages. pH is determined by the activity of lactic acid bacteria (LAB) during the initial phase of ensilage fermentation (Davies et al, 1998). As silage pH is not constant throughout fermentation, further investigations into the effect of pH, on metacercarial viability should include looking at the effect of pH change over time, especially in the initial phases of ensilage. As acid content is related to silage quality, investigating the effect of different concentrations of the respective lactic, acetic, butyric and propionic acids on metacercarial viability may also be informative.

From this experiment, it can be concluded that the pH associated with silage fermentation is not, in itself, a significant factor in metacercarial survival. However, repeat experiments with larger group sizes would be required to determine if lactic acid and or combinations of lactic, acetic and butyric acid does degrade cyst walls and consequently reduce the viability of metacercariae. An assessment of cyst walls can be made visually with a microscope but as discussed before, in Chapter 2, this is not a good method for determining metacercarial viability. Fluorescent based microscopy which utilises the absorption of dyes depending if a parasite is dead or alive could be an effective alternative to visual assessment of cyst walls by light microscopy. Peak, Chambers and Hoffmann (2010) developed an fluorescent based bioassay to determine the viability of *Schistosoma* larvae. The dye fluorescein diacetate (FDA) crosses the membranes of living cells. And propidium iodine (PI) cannot permeate the membranes of live cells and can only stain nucleic acids if the integrity of cell membranes is degraded by the parasite dying. It is thought that this method could be adopted in future work on metacercarial viability and the effect of acids on cyst walls.

Metacercariae are highly susceptible to desiccation (Boray & Enigk, 1964). This may explain the poor recovery and excystment rates of metacercariae from the ensilage experiments. Moisture levels are important to metacercarial survival. This was demonstrated by Boray and Enigk (1964). Their experiments showed that, at 10 °C in 75-80% relative humidity, no metacercariae were viable after 50 days but at 90% relative humidity, at the same temperature, 82% of metacercariae were viable after 112 days (Boray & Enigk, 1964).

The importance of moisture, in the context of the DM content of silage, for metacercarial survival is demonstrated in this project. The DM content of the ‘high’ quality silage was an average of 321 g/kg (32.1%). After 4 weeks, 14 of the original 150 metacercariae were viable from the ‘high’ quality silage but 75 out of 150 metacercariae from the complementary control were viable. The moisture levels of the silages were perhaps not conducive to long-term survival of metacercariae. Metacercariae have been found to cause a light infection in animals after heavily contaminated hay, which had not been dried sufficiently, was fed after 1-3 weeks storage in a stable. However, no infection was observed when the same hay was fed after 5-6 months in storage (Nöller & Schmid, 1929, as cited in Boray & Enigk, 1964). The results of the experiment performed in this study support the theory that metacercariae can survive a short time in silage. Having said that, other publications do not support this theory. For example, *F. hepatica* metacercariae did not survive 35-57 days in silage (Wikerhauser & Brglez 1961, cited by Dawes 1965). Similarly, Tarczynski and Podkowka (1964) did not find evidence of fasciolosis in rabbits after they were fed silage in which metacercariae had been present for just 1 day. It should be noted that although the silage used for this experiment was sampled from farm clamps the conditions of the small bales created with this silage in the lab is not fully representative of the conditions on-farm.

Naturally, it would be expected that the conditions of silage fermentation and the way in which silages are made vary greatly, this may account for the differences in the evidence previously reported. From the experimental conditions explored in this project, it may be concluded that the moisture level of the ‘high’ quality silage was sufficient to support metacercarial survival for ~4 weeks. The moisture content of the ‘medium’ and ‘low’ quality silages was higher (average DM content of 27.1% and 26.1 %, respectively), so it is possible that metacercariae did survive 4 weeks in those silages. However, as no data was collected for metacercarial viability at 4 weeks for these silages, metacercarial survival can only be surmised. A repeat study should include a larger experimental design, including equal sets of each silage type, multiple time points and multiple replicates for better comparisons of metacercarial viability.

Farmers will typically aim for ~30% DM in their silages. Harvest is ideally performed in dry weather conditions to allow the grass to wilt before storage. Poor quality silages can be the result of insufficient wilting before storage and/or harvesting in wet conditions. If the DM of these crops is compromised in this way (i.e. a wetter crop), this will lead to poorer fermentation and loss of nutritional value during storage of the silage (Borreani et al., 2018). Harvesting silage in wet conditions may increase the risk of the grass being contaminated with metacercariae as the conditions will be more suited to the activity and survival of *G. truncatula* and liver fluke life stages on pasture. Some western wetter farms, where the DM% of silage can be 20% or less (Davies et al., 1998), could be at a higher risk if they have an established fluke life-cycle on the farm. Particular fields or areas of fields will be higher risk than others as the environment of a farm is not identical over all the land. The source of the grass ensiled, either a particular field or areas of fields, will be important in understanding the risk of liver fluke metacercariae in the silage.

Temperature is another important factor in metacercarial survival. The higher the temperature of the environment the more rapid the loss of metacercarial viability (Boray & Enigk, 1964). All silages were incubated at room temperature (~21°C) for the duration of the experiment compared to the pH experiment which was performed at 4°C. Boray and Engik (1964) demonstrated that temperature has the ability to halve the percentage viability of metacercariae. They found that 86% of metacercariae were viable after 130 days when incubated at 10° C but only 46% for the same amount of time when incubated at 20°C (Boray & Enigk, 1964). The experiment performed for this chapter shows a similar story when comparing the control groups of metacercariae. As part of the experiment looking at the effect of pH 4 over time, the viability of the control group of metacercariae stored in water at 4°C for 16 weeks was 72%. In contrast, the viability of the control group of metacercariae stored in water at 21°C for 16 weeks as part of the metacercarial ensilage experiment was 7%.

As is common in the climate of the UK, higher temperatures usually results in drier conditions. The combined effect of low moisture and high temperatures will more rapidly result in metacercarial desiccation. This is again demonstrated by the experiments of Boray and Enigk (1964). After 14 days at 10°C, relative humidity of 90%, 80% of metacercariae were viable. The viability was 32% at 75%-80% humidity at the same temperature for 19 days but when the temperature was 20°C (75-80% humidity) the viability of metacercariae at 14 days was 5% (Boray & Enigk, 1964). They concluded that metacercariae could survive storage in hay under winter conditions provided that the hay had not been properly dried (Boray & Enigk, 1964). It may be understood from these findings that in order to reduce the risk of viable metacercariae in silage and or hay, farmers should harvest during days of high temperatures, avoiding days just after rain or days of rain.

A fundamental flaw of the current experiment conducted as part of the work for this chapter is the fact that metacercariae were introduced to the silages after the initial first stage of fermentation was already underway. ‘Real’ silage samples were taken from farms, these silages, regardless of quality, had stabilised in their fermentation at the time of sampling. Spiking the silages was done after the initial fermentation in the hope of making the process of recovering any metacercariae easier. Crucially, the metacercariae were not exposed to the dramatic changes in pH and temperature that occur during initial silage fermentation. A more reflective study of metacercarial survival in silage would incorporate the use of grass, purposefully contaminated with metacercariae from infected snails, to make silage. It was unknown if the silages sampled already contained *F. hepatica* metacercariae. To remove the possibility of metacercariae in silage samples experimental grass could, in future experiments, be grown and harvested on snail free pasture or in a laboratory.

The results of the experiment performed with vacuum-packed bags of silage gave an idea of how metacercariae might survive in bales or clamps of silage on farmers. One important distinction between these two scenarios is that metacercariae were not exposed to all the phases of making silage. Typically, there are four phases to the process; (1) the aerobic phase, (2) the fermentation phase, (3) the storage phase and (4) the feed-out phase (Borreani et al, 2017). The conditions between each phase can vary greatly, especially form the initial harvest of the grass to the aerobic phase just after sealing the silage pit or bale. The most critical to metacercarial survival would be the time just after harvest. To ensure the best DM content possible, farmers will wilt their grass before storing in a silo or wrapping grass into silage bales. F As previously stated, metacercariae are vulnerable to desiccation, so it is possible that during the wilting phase, many of the metacercariae encysted on the grass could lose viability at this point. It is also during this phase that the biggest change in conditions will arise. The metacercariae in our experiment missed the initial rise in temperature, takeover of lactic bacteria and decrease in oxygen that would happen at the beginning of the silage fermentation process. This might be the most critical stage in the survival of metacercariae and may explain why the rabbits used by Tarczynski & Podkowka (1964) did not subsequently become infected, rather than the pH, as originally assumed.

The success of grass silage fermentation relies on many complicated factors. Therefore, it is difficult to discern the possible risk of metacercarial survival in silage crops. Despite this, the conditions required for silage fermentation do not favour the survival of many aerobic organisms. Listeria, yeasts, bacilli and moulds are all examples of organisms that can cause aerobic spoilage of grass silage and are also, like liver fluke, detrimental to animal health (Oude Elferink et al., 2000). Without any actual evidence, it has been widely assumed that the high concentration of carbon dioxide and high temperatures involved in fermentation would cause damage to metacercarial cysts (Kendall, 1965).

Silage harvesting and ensilage processes do differ across UK farms. Grass grows faster in warmer climates and this is demonstrated by earlier harvests in the South of England compared to farms in Scotland (average of 1 month earlier). Differences in ensilage methods might include sugar content of the harvested grass, length of wilting, the use of additives and type of storage (bale vs clamp). Despite these differences, farmers all want to achieve optimal fermentation of their grass crop to ensure the best quality silage to meet the nutritional needs of their stock. This can be difficult with the reality of inconsistent weather. For farmers, it is often a race against time to get silage cut, wilted and sealed before it starts to rain. Rain can make it difficult to reach the optimal DM content for silage as often there is not sufficient time to wilt the chopped grass before ensilage. Wetter weather also favours the life-cycle and activity of *G. truncatula* and *F. hepatica,* respectively. Where the habitat of infected *G. truncatula* and silage fields intersect, there is a high chance that grass will be contaminated with metacercariae. It is predicted that climate change will result in wetter warmer weather (Kenyon et al., 2009). It has been suggested that the rate of drying silages in the field will increase but this will also coincide with an increase in poorer silages or ‘silage spoilage’ because of the increase in wetter weather (Cooper et al., 1996). Spoiled silages will have lower DMs and, as it was demonstrated that metacercariae can survive silage with a DM of 32.1% for 4 weeks, it is theoretically possible that metacercariae could survive in silages of low DM for longer.

The recovery of metacercariae from silage was challenging and the numbers of metacercariae recovered were poor and inconsistent. Ultimately, the success of metacercarial recovery has affected the results of the experiment. A greater recovery rate may have resulted in viable metacercariae being detected past 4 weeks. The issue raised in this Chapter and in Chapter 3 is that silage and grass are difficult substrates to isolate metacercariae from.

It was difficult to process the large volume of silage material. Ensuring that every surface of the grass was washed was important in order to release and re-suspend the metacercariae in water. This would allow for the collection of the metacercariae when sieving the material. While washing the silage, it is possible that the metacercariae were somehow lost within the silage or were washed away during the process. The real challenge was identifying the metacercariae from the sieved material under the microscope. The metacercarial cyst walls are brown in colour and so can easily blend into the colours of the materials collected on top of the sieves. Identifying metacercariae under these conditions was very difficult, requiring a lot of practice and patience.

A possible alternative to the painstaking identification of metacercariae from plant material using a microscope would be the use of centrifugation and density gradients. This type of isolation was successful using Percoll and Metrizamide (Fry et al., 1985). This method claims the ability to isolate metacercariae from plant material and from metacercarial cyst walls. Although the inner cyst is missing, empty cyst walls can be mistaken as whole cysts. The isolation of cysts walls from metacercariae is advantageous as it removes the chance of error by the lab technician. The use of isolation by density gradient could be incorporated into the a repeat experiment. This could be performed on the washed, sieved plant material thus reducing the time required to identify, isolate and count metacercariae recovered from the silage material.

Reports of fasciolosis in stock housed year-round may be because of contaminated feed or it could be the result of an active liver fluke life-cycle somewhere in the housing. It is possible that infected *G. truncatula* snails are present around water supplies and could be shedding cercariae into the water. The areas immediately around water troughs can make good habitats for *G. truncatula* (Knubben-Schweizer & Togerson, 2015). Cercariae are able to encyst on surface of water (Abrous et al., 2001) and the metacercariae will float on the surface until ingested by an animal. Similar to the management of fields to reduce fluke risk, ensuring good drainage around water trough areas can help reduce and remove habitat suitable for snails.

The perceived risk of liver fluke infection from grass silage cannot be definitively ruled in or out based on the work performed here. There are, however, steps that farmers can take to reduce risk of metacercariae in their silage. Proper drainage of silage fields will help remove and reduce the habitat suitable for *G. truncatula.* Following common rules about silage making can also help reduce the risk of incorporating viable metacercariae in forage samples. Cutting and harvesting silage when the grass is not wet (aim for high DM) and wilted sufficiently will increase the chances that any encysted metacercariae die from desiccation before or during ensilage. Similarly to preventing the growth of unwanted spoilage microorganisms farmers should ensure that silage is tightly sealed to promote anaerobic conditions and good fermentation. The *F. hepatica* life-cycle can also be managed from the point of view of the definitive host by testing stock regularly. Performing faecal egg counts to check for adult liver fluke in stock and then responsibly using appropriate flukicides to remove the parasites is a good place to start. Understanding the current status of liver fluke on a farm, the various diagnostic and control options available is also important in tackling this parasite sustainably.

More evidence of metacercarial survival in silage is needed for a more comprehensive understanding of the topic. Further investigations should include a controlled trial utilising grass, deliberately contaminated with metacercariae from infected snails, prior to ensilage. Recovery of metacercariae and subsequent excystment would be beneficial for reducing the use of model animals, however, feeding the contaminated silage to definitive host sheep, for example, would not only allow us to show if the metacercariae are viable but also if they are capable of causing clinical disease after being ensiled.

4.5 Conclusion

It is difficult to answer the question of metacercarial survival in grass silage definitively. Very little information has been published on this subject to date. Tenuous links to this topic can be made through a few older publications. However, these studies use rabbits as experimental hosts, which are fed hay infected with metacercariae to study the appearance of fasciolosis, are very far removed from the present problem of fasciolosis risk to livestock fed grass silage. There are many factors that contribute to the fermentation process of grass and the factors of fermentation success are also complicated. Therefore, it is difficult to discern the survival of metacercariae in silage crops. The multifactorial and variable nature of silage fermentation further complicates the understanding of what fermentation conditions may allow for metacercariae to survive in grass silage.

Although the real risk of fasciolosis to livestock grazing ensiled grass is still unknown, this Chapter has shown that *F. hepatica* metacercariae can survive pHs associated with grass silage. The experiments have also shown that metacercariae can survive a small amount of time, 4 weeks, in ensilage conditions. Ultimately, pH is not likely to be a significant influence on metacercariae survival. The DM content of silage and the temperature of the silage during fermentation are likely to be the most important determinates of metacercarial survival and risk of fasciolosis from silage.

Chapter 5: Development of DNA assays for the identification of *F. hepatica* metacercariae from pasture samples

5.1 Introduction

Currently liver fluke risk in the UK is determined using the Ollerenshaw index (NADIS, 2019). Data of liver fluke prevalence, measurements of rainfall, the number of rain days and the potential evapotranspiration are used to produce a forecast of regional liver fluke risk (NADIS, 2019). Faecal eggs counts from livestock, numbers of liver condemnations, snail numbers, the number of infected snails and total of disease cases were used to validate the forecast index (Ollerenshaw, 1974). The Ollerenshaw index cannot accurately predict the risk of liver fluke for current farms as it was originally applied for farms and a climate of the past. The climate and liver fluke epidemiology have changed since this index was first developed in the1960’s (Kenyon et al., 2009). The habitat suitable for *G. truncatula* has expanded due to milder, wetter weather and now areas previously perceived to be fluke free i.e. the eastern drier areas of the country are reporting cases of fasciolosis (Mitchell, 2002; Kenyon et al., 2009 and Howell, et al., 2015). Another fault with this method of liver fluke forecasting is the absence of data relating to the presence of metacercariae on pasture. Data relating to snails and climatic data relating to the survival of snails and eggs on the pasture can only give a crude estimation of liver fluke risk.

Sheep and cattle become infected when they ingest grass coated with encysted *F. hepatica* metacercariae. The presence and abundance of liver fluke metacercariae on pasture is the ultimate indicator of disease risk to sheep and cattle. It is, therefore, logical that to prevent fasciolosis in their flock and herds, farmers should avoid grazing livestock on likely contaminated pasture at high risk times. Traditionally, late summer and autumn are the periods of highest risk in the UK (Ollerenshaw & Rowlands, 1959). The infectious metacercariae are likely to be at their most viable and abundant on the pasture at this time.

The most challenging aspect of understanding fluke risk is determining when and for how long the highest risk will occur. A routine diagnostic test for the detection of *F. hepatica* metacercariae from pasture does not exist.

Traditionally, the presence of *F. hepatica* infected *G. truncatula* in a field or in an area of a field was used as an indicator of potential fluke risk. Most of the time farmers already suspect fields or areas of pasture where their animals are becoming infected because high risk areas are identifiable, usually, by the presence of *Juncus sp.* reeds and water logged ground. Indeed, in the years when the climatic conditions are favourable to the survival of *G. truncatula* the collection of snails is fairly straightforward. However, searching for snails in fields is difficult work and often time consuming (Charlier et al*.,* 2011). Many environmental and human factors influence the success of finding snails.

Despite the difficulty of detecting *G. truncatula* in the environment, detecting metacercariae on pasture poses an even greater challenge. The Ministry of Agriculture published a mechanical method for the recovery of metacercariae from pasture samples. This method involves the washing of sampled grass through metal sieves, then utilises the oxidative effect of sulphuric acid to shrink any organic matter in order to improve the detection of metacercariae from other materials (Ministry of Agriculture, 1986). This method boasts a recovery rate of up to 90% but it is not performed routinely, even by researchers.

An alternative to the time consuming task of manual identification of metacercariae from pasture, is the detection of DNA targets from pasture samples. DNA methods are already used in research laboratories as diagnostic tools for the identification of *F. hepatica* from faecal samples and from snails collected from fields. Polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) are the main DNA based assays used to identify parasite stages targeting the cytochrome oxidase subunit 1 (COX1). Relative to other diagnostic tests, PCR and LAMP are easy to interpret, with the appearance of corresponding DNA base pair band or fluorescence as the determination of a positive sample. These tests are also designed to be highly sensitive, detecting a particular target at very low concentrations. This is beneficial when using environmental samples as DNA originating from life stages of *F. hepatica* will be present in comparatively minute quantities.

COX1 is a highly conserved sequence of mitochondrial DNA. It is used widely within genetic taxonomy to determine the identity of closely related species (Hebert et al., 2003). PCR assays amplifying targets of this gene have already been established as reliable diagnostic tests for fasciolosis in sheep. *F. hepatica* DNA can be detected in the faeces of sheep 3 weeks post infection with no cross contamination with DNA of other trematodes (Martìnez-Pèrez et al., 2012).

LAMP requires one constant temperature (~63 °C) for DNA amplification as isothermal polymerases, such as Bst, are used in LAMP reactions to displace DNA strands (Notomi et al., 2000). Effectually, LAMP does not require denatured DNA template for amplification, unlike PCR and can amplify a large amount of DNA very quickly (~45 minutes) without thermocyclers (Nagamine, 2002). Bst polymerases have a better tolerance than Taq polymerases to inhibitory components of biological samples such as blood and faeces (Poon et al., 2006). LAMP reactions boast strong specificity to their DNA target as this method uses 4 primers sets which can identify a total of 6 individual sequences of the DNA target (Sotiriadou & Karanis 2008). LAMP has been shown to have greater sensitivity than PCR. Ai et al., (2010) demonstrated a LAMP assay, targeting the ribosomal intergenic spacer of *F. hepatica,* that was 10^4 times sensitive than it’s PCR counterpart. All of the characteristics of LAMP described indicate promise in the successful application of this method to the detection of *F. hepatica* metacercarial DNA from pasture samples.

This Chapter aims to demonstrate the use of both PCR and LAMP for the detection of *F. hepatica* metacercariae from samples of DNA extracted from grass spiked with *F. hepatica* DNA and from grass spiked with metacercariae. This Chapter also aims to evaluate the possible application and translation of these tools for forecasting fasciolosis on farms and in the environment

5.2 Materials and Methods

5.2.1 Sample preparation

Metacercariae

F. hepatica metacercariae (strain Gloucester, shed date unknown, Ridgeway Research, UK) were stored in sterile distilled water at 4°C on cellophane sheets within 10 ml capacity tubes. Maintenance involved the addition of fresh sterile water to the tubes every month.

Grass

Lolium perenne seeds (Homebase, UK) were sown and grown as described in Chapter 3 (section 3.2.6). This ensured that the grass was free of DNA from *F. hepatica*. The grass was harvested after a month of growth cutting as close to the bottom of the stems without including soil in the samples.

Grass spiked with metacercariae

Twelve samples of freshly cut grass, 0.05g, were spiked with a single *F. hepatica* metacercaria each. These samples were used in both PCR and LAMP experiments, for the amplification of the *F. hepatica* COX1 sequence.

1g of freshly cut grass was spiked with 100 metacercariae. This mixture was processed using a blender (Philips, Netherlands) together in 100ml of water for 1 minute. The puréed contents was strained using a 100um metal sieve. The material remaining on top of the sieve was split into 12 subsamples weighing 0.05g for DNA extraction.

Samples of freshly harvested grass, cut using sterile scissors, weighing 0.05g were processed for DNA extraction separately for use as no target controls and serial dilutions.

5.2.2 DNA extraction

All DNA samples, including *F. hepatica* metacercariae and grass samples, were extracted using the DNeasy® Blood and tissue extraction kit (Qiagen, Germany). DNA was extracted from metacercariae according to the number required for the experiment. The protocol for DNA extraction was followed as per manufacturer's instructions but DNA was eluted in 100 µl of Buffer AE rather than 200 µl. Full description of protocol can be found in Appendix 1.

5.2.3 *F. hepatica* DNA dilution series

The sensitivity of PCR and LAMP was determined using serial dilution of *F. hepatica* adult DNA diluted in water and in DNA extracted from grass. 10 µl of *F. hepatica* DNA was serially diluted 1:10 in nuclease free water to give set of samples with a concentration gradient of 1-1×10^{-6} ng/µl. The same serial dilution conditions of *F. hepatica* DNA were created, 1:10 but using DNA extracted from grass.

5.2.4 DNA purification

DNA purification was performed on samples of DNA extracted from grass, DNA extracted from grass spiked with metacercariae.

Ethanol precipitation

10µl of 3M sodium acetate and 300µl of ethanol (100%) was added to DNA samples and stored at -20°C for 1 hour. Mixtures were centrifuged at 13000rpm at 4 °C for 30 minutes and the supernatant removed. The remaining pellet was washed with 500µl ethanol (70%). The mixture was centrifuged at 13000rpm for 15 minutes at 4°C and the supernatant removed. The remaining pellet was allowed to air dry on the lab bench before re-suspending in 50µl of molecular grade water. This method was provided by Seamus Stack (MAST).

QIA Quick PCR purification

Protocol was followed as per manufactures instructions (Qiagen)

5.2.5 Polymerase chain reaction (PCR)

The COX1 gene was chosen as the target for amplification. This is a highly conserved region of DNA and its use in taxonomy studies allows for identification and discern between closely related animals (Hebert et al., 2003). *F. hepatica* COX1 PCR was performed in 20 µl reaction volumes containing: 10x reaction buffer (Invitrogen, USA), 50mM $MgCl_2$ (Invitrogen, USA), dNTP mix (Invitrogen, USA), Nuclease free H_2O (Sigma) and 5 U Platinum *Taq* polymerase (Invitrogen, USA). The *F. hepatica* mitochondrial DNA fragment FhCox1 was amplified from metacercarial DNA using Cox 1 F 5' –GTTGGCATATTGCGGCTTAG-3' and Cox 1 R 5'- AGGGATCTGCACCTCAACTC-3' primers (Martínez-Pérez et al., 2012). Cycling conditions were as follows: 2 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 63°C for 30 seconds, extending at 72°C for 45 seconds and finally 10 minutes at 72°C (Biometra, Thermocycler gradient, Germany). PCR products were analysed on a 2% agarose gel prepared using Tris-acetate EDTA (TAE) and stained using GelRed™ Nucleic acid stain (Biotium). Gels were run at 100v for and visualized on AlphaImager™2200 (Alpha Innotech).

5.2.6 Sequencing

Select DNA samples positive for the *F. hepatica* COX1 using the Martínez-Pérez et al., (2012) primer set were chosen for direct nucleotide sequencing. PCR products were tested for DNA quantity and purity on NanoDrop 1000 spectrophotometer (Thermos Scientific, USA). According to the measured concentration, PCR products were diluted in the required amount of nuclease free water to give a sample concentration of 5ng/µl and sample volume of 15 µl. 2 µl of the forward COX1 primer (10 pmol/µl) was added to the samples. Sequencing was performed by Eurofins MWG Operon (Germany). DNA sequence data was analysed using the MEGA6: Molecular Evolutionary Genetics Analysis (Version 6.0) programme and compared against sequences in the NCBI (National Centre for Biotechnology Information) data base using the BLAST programme: https://blast.ncbi.nlm.nih.gov/Blast.cgi

5.2.7 Loop mediated isothermal amplification (LAMP)

LAMP assays were performed using lyophilized LAMP pellets and kits provided by MAST (UK). V6.21, V6.31 and SG4.54 reagent pellets (MAST, UK) were used throughout the project. The primer mix was made up using *F. h* COX 1A primer set (MAST, UK) and volumes displayed in Table 5.1.

Table 5.1 – Primer sequences and volumes for *F. hepatica* LAMP assay

Primer	**Sequence (5'-3')**	**Volume (µl)**
F3	TTTGTAAAGCAGAGGTGGTT	0.5
B3	ACCGAGGAAGACCAAGAA	0.5
Fip	TCCGAATCACCTATCACAACCTGGTTTGGATTGATTGTTTCCG	4
Bip	GGCATATTGCGGCTTAGTTATGACAGAGCCACAAACGAAT	4
F Loop	AACAACCCTAAGCAAGCAAA	1
B Loop	GGTCGTTGGTAAGATCAGGATT	1

V6.21, V6.31 pellets were rehydrated in 86 µl of 0.1M Tris buffer. A master-mix was made; for every reaction, 8.6 µl of buffer and 0.4 µl of primer mix were added. 9 µl of master mix was used per reaction adding 1 µl DNA sample or 1 µl of nuclease free water for the negative control. The positive control was an in house 10ng/µl sample of adult *F. hepatica* DNA or a synthetic positive control provided by MAST within the LAMP kit.

MAST ISOPLEX® *DNA* LYO kit (MAST, UK) contained the SG4.54 pellets. These were reconstituted in 20µl 0.5M Tris and 68µl of molecular grade water. Synthetic positive controls included in the kit were dissolved in 50µl of molecular grade water for 5 minutes. Positive control primers, provided by MAST, were reconstituted in 20 µl of molecular grade water. Unless specified, assay reactions were set up as per the manufacturer's instructions.

Four LAMP assays deliberately deviated from manufacturer's instructions. These assays used SG4.54 pellets. Reconstitution of the pellets, for these four assays, was performed using 2µl 0.5M Tris and 84µl molecular grade water.

The amplification of DNA by LAMP was measured on a real-time PCR machine 7500 (Applied Biosystems, USA). Reactions were performed at 63°C and measuring the fluorescence every 33 seconds (99 cycles) on the FAM filter.

Positive detection of the *F. hepatica* COX1 (MAST) target was determined by the detection of fluorescence by dye FAM before the cut-off time of 40 minutes.

For assays that included the use of the V6.21 and V6.31 pellets the baseline was set at 3-15 cycles. For reactions using the SG4.54 reagent pellets the baseline of the assays was set at 3-5 cycles. The threshold for each assay was set individually.

5.3 Results

5.3.1 PCR

F. hepatica dilution series, as described in section (3.2.2), was used in a PCR with the *F. hepatica* COX1 primer set (Martínez-Pérez et al., 2012). Amplification of the target (~423bp) was observed in DNA concentrations 10 ng/µl to $1\text{x}10^{-4}$ ng/µl but not for DNA concentrations $1\text{x}10^{-5}$ or $1\text{x}10^{-6}$ ng/µl (Figure 5.1). The same dilution series was set up in DNA extracted from grass. The target was only detected in concentrations of 10 ng/µl, 1 ng/µl and 0.1 ng/µl *F. hepatica* DNA (Figure 5.2).

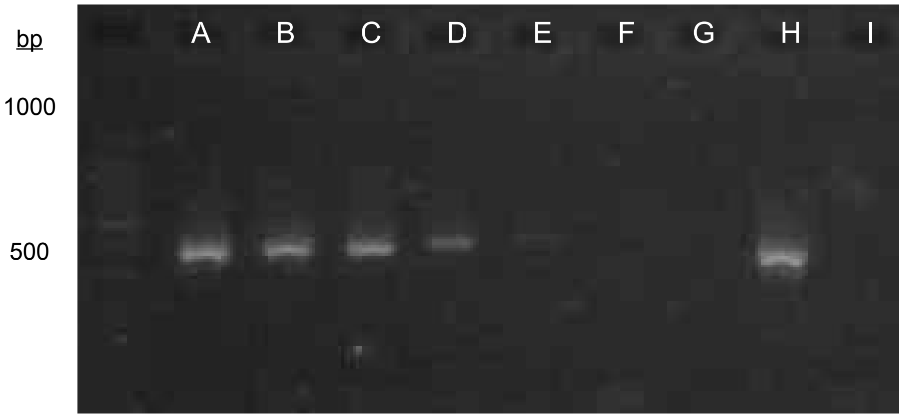


Figure 5.1 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A to G = 1- $1x10^{-6}$ ng/µl dilution series, well H = 10 ng/µl, well I = NF H_2O. 100bp DNA ladder (Invitrogen)

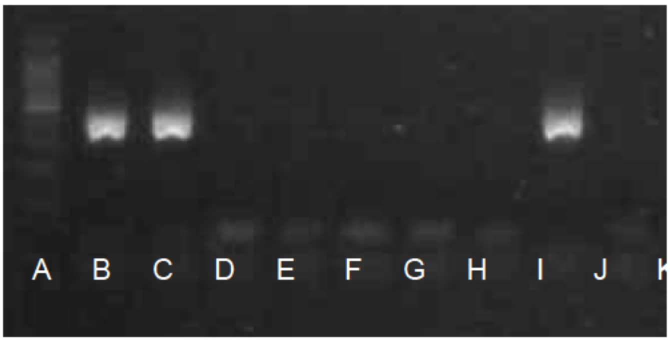


Figure 5.2 - Gel image of *F. hepatica* DNA amplification with F. h COX1 (Martìnez-Pè ez et al., 2012) primer set, ~423bp target. *F. hepatica* DNA diluted in DNA extracted from grass. Wells A to G = 1- $1x10^{-6}$ ng/µl dilution series, well H = 10 ng/µl, well I = NF H_2O. 100bp DNA ladder (Invitrogen)

bp
1000
500

The *F. hepatica* COX1 target was not observed in DNA extracted from samples of purified DNA extracted from grass; samples A-D (Figure 5.3). The target was detected from DNA extracted from 5, 10, 20, 30 and 100 metacercariae but not from DNA extracted from a single metacercaria (Figure 5.3). This suggests that the concentration of *F. hepatica* COX1 target was not high enough to be detectable from the DNA sample extracted from the single metacercaria or that DNA extraction had failed for this sample.

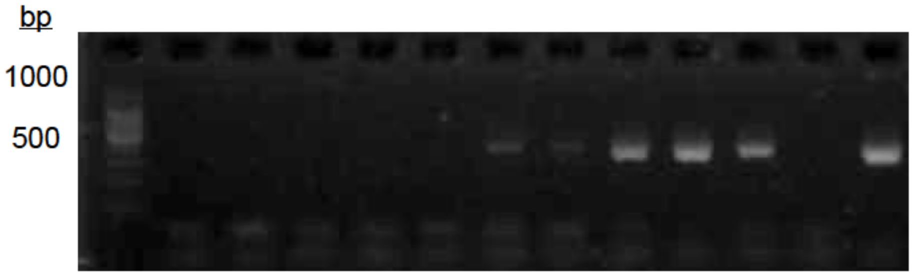


Figure 5.3 - Gel image of *F. hepatica* DNA amplified with F. h COX1 Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A-D = purified grass DNA, Wells E-J = DNA extracted from 1, 5, 10, 20, 30 and 100 metacercariae, well K = NF H_2O, well L = 10ng/µl. 100bp DNA ladder (Invitrogen)

bp
1000
500

Out of the seven DNA samples (D-J) extracted from grass, previously spiked with DNA from ~280 *F. hepatica* metacercariae, the COX1 target was amplified from four (D, E, H and J). The target was not detected from DNA extracted from just grass DNA (A-C) (Figure 5.4).

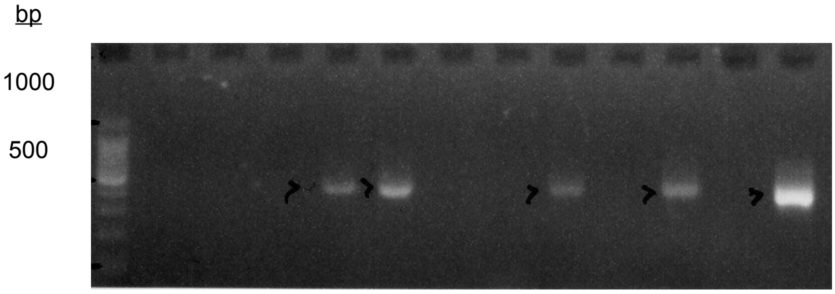


Figure 5.4 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A-C DNA extracted from grass, wells D-J = DNA extracted from grass spiked with DNA extracted from ~280 metacercariae, well K = NF H_2O, well L = 10 ng/µl. 100bp DNA ladder (Invitrogen)

The same seven samples spiked with metacercarial DNA were tested again for the *F. hepatica* COX1 target after DNA purification. Samples E and H remained positive for the target after DNA purification (Figure 5.5). Sample I was positive for the target after purification where previously it had not been (Figure 5.4). Previously positive *F. hepatica* COX1 samples, D and J, were not positive after purification. Purified DNA extracted from grass did not result in detection of the *F. hepatica* COX1 target by PCR (samples M-R) (Figure 5.5).

Figure 5.5 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells D-J = purified DNA extracted from grass spiked with ~280 metacercariae, wells M-R = purified DNA extracted from grass, well K = NF H_2O, well L = 10 ng/µl. 100bp DNA ladder (Invitrogen)

Freshly cut grass was purposefully spiked with 100 *F. hepatica* metacercariae. Twelve subsamples were created from this grass sample. PCR amplification of the *F. hepatica* COX1 target on these subsamples revealed two out of the twelve subsamples (G and I) were positive for the target (Figure 5.6).

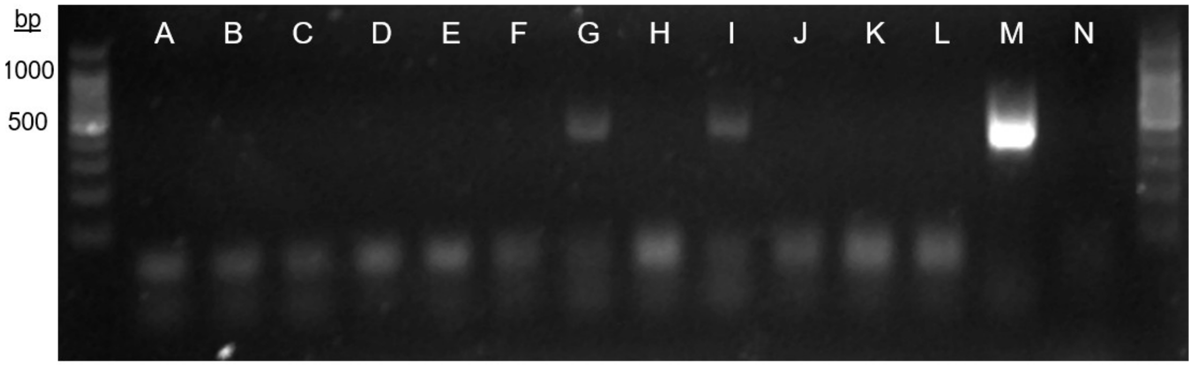


Figure 5.6 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A-L DNA extracted from subsamples (0.05g) of grass spiked with 100 cysts, well M = 10ng/µl, well N = NF H_2O. 100bp DNA ladder (Invitrogen)

Eleven individual grass samples (0.05g) were spiked with a single metacercaria each before DNA extraction. Five samples (B, C, F, G and I) were positive for the *F. hepatica* COX1 target after PCR amplification with the Martìnez-Pèrez et al., (2012) primer set (Figure 5.7).

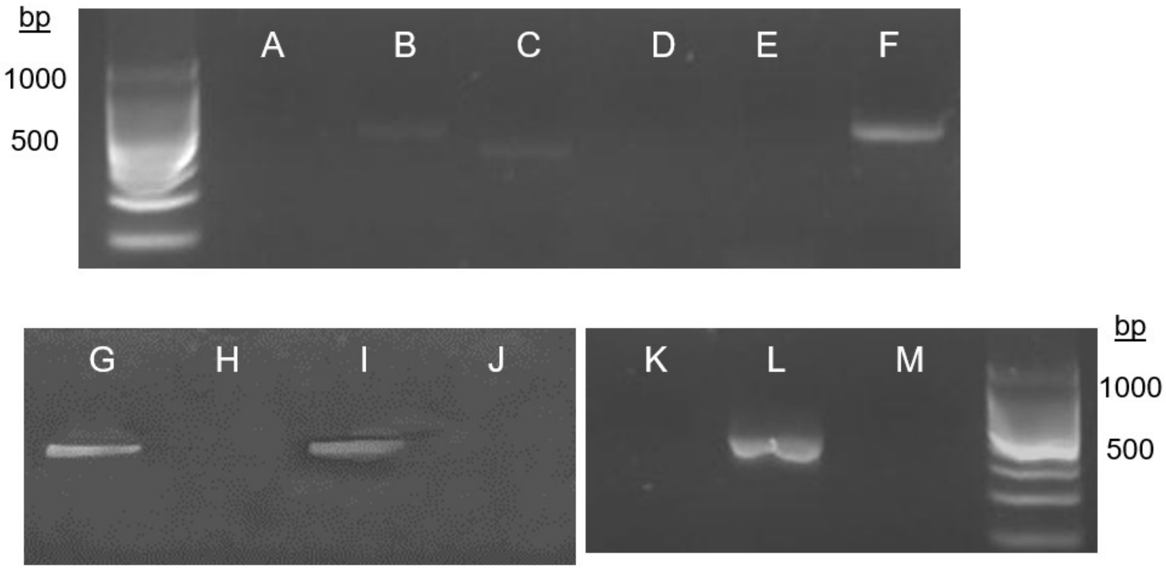


Figure 5.7 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A-K DNA extracted from subsamples (0.05g) of grass spiked with a single cyst, well L = 10 ng/µl, well M = NF H_2O. 100bp DNA ladder (Invitrogen)

5.3.2 Sequencing

The amplification products of PCR with *F. hepatica* COX1 target from samples of 0.05g of grass containing either thirty metacercariae or a single metacercariae were sent to Eurofins MWG Operon (Germany) to be sequenced. The return from running sequences in BLAST (done by author) is displayed in Table 5.2. Accession number AP017707.1 belongs to a sequence titled- *Fasciola hepatica* mitochondrial DNA, complete sequence. Four samples had high similarity to this sequence. PCR products also returned high similarity to accession number KU058264.1, belonging to *Fasciola hepatica* isolate Egypt/BSU-2 tRNA-Thr mitochondrial linear DNA. The similarity of amplified sequences to the returned accession numbers indicates that the COX1 primers are amplifying mitochondrial DNA of *F. hepatica* from samples of grass DNA previously spiked with either thirty metacercariae or a single metacercaria.

Table 5.2 – GenBank BLAST results on PCR products from amplification with F. h COX1 primer set (Martìnez-Pèrez et al., 2012)

Samples	No of metacercariae in grass sample	Sequence length (bp)	Accession number	% identity
A	30	383	AP017707.1	99
B	30	385	AP017707.1	99
C	1	295	AP017707.1	100
D	1	381	KU058264.1	99
E	1	43	AP017707.1	97
F	1	12	Too small to BLAST	-
G	1	380	KU058264.1	99

5.3.3 LAMP

Amplification data from all LAMP assays was collected on a real-time PCR machine. The software affiliated to the machine displays amplification of the target as a linear line graph of fluorescence emitted by an intercalating fluorochrome dye (Figure 5.8). In order to make the process of determining which samples are positive for the *F. hepatica* COX1 target easier, the CT values generated for each assay were converted into times to detection using this equation; (CT x 33) ÷ 60.

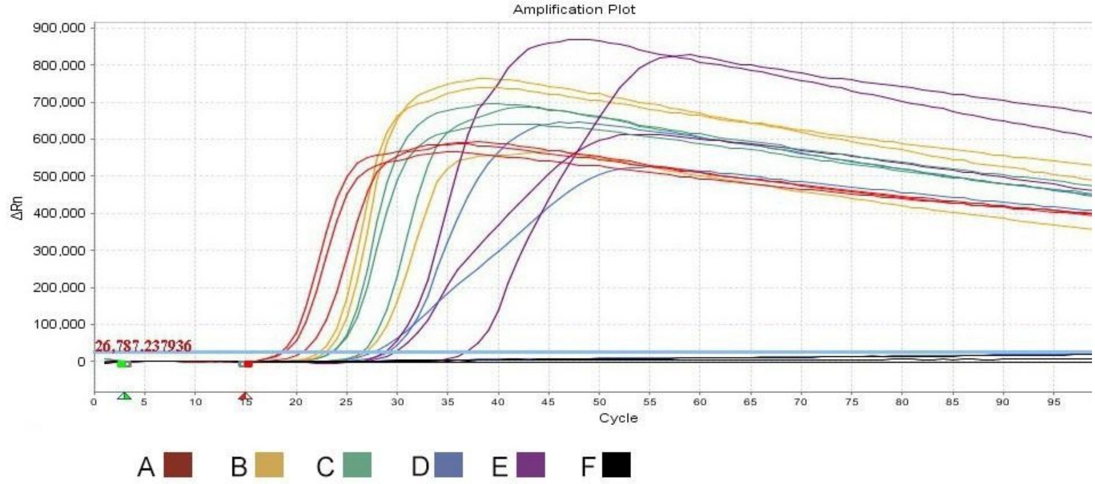


Figure 5.8 - Line graph generated by ABI 7500 software depicting ΔRn or fluorescence by cycle. The baseline for this assay was set at 3-5 cycles and the threshold is displayed in red. Those samples that produce a line above the threshold are considered postive. Each sample was tested in triplicate. A= 10 ng/µl *F. hepatica* DNA, B-D DNA extracted from 10, 20 and 30 *F. hepatica* metacercariae, respectively. E = DNA extracted from 1 *F. hepatica* metacercariae. F= NF H_2O

As an example, the above graph (Figure 5.8) has been converted into an individual dot plot of times to detection using Minitab 17 (Figure 5.9). A table in appendix 3 displays the CT values and corresponding time to detection for each value for each assay. LAMP successfully detected the target from all samples of DNA by or before twenty minutes. All blue dots presented below the red line represent positive detection of the *F. hepatica* COX1 target before the forty minute cut-off. The blue points above the red line represent negative samples for the *F. hepatica* COX1 target. The time taken to detect the target appears to be slower from DNA samples extracted from one metacercaria compared to DNA sample extracted from ten metacercariae. This trend does not continue with DNA samples extracted from larger numbers of metacercariae. Times to detection from DNA extracted from ten, twenty and thirty metacercariae cluster in similar patterns around fifteen minutes into the assay. The *F. hepatica* COX1 target was not successfully amplified from one replicate of DNA extracted from thirty metacercariae. The target was successfully detected from all replicates of the positive control; 10ng/µl of *F. hepatica* DNA, clustering around ten minutes. The target was not detected from the no target control.

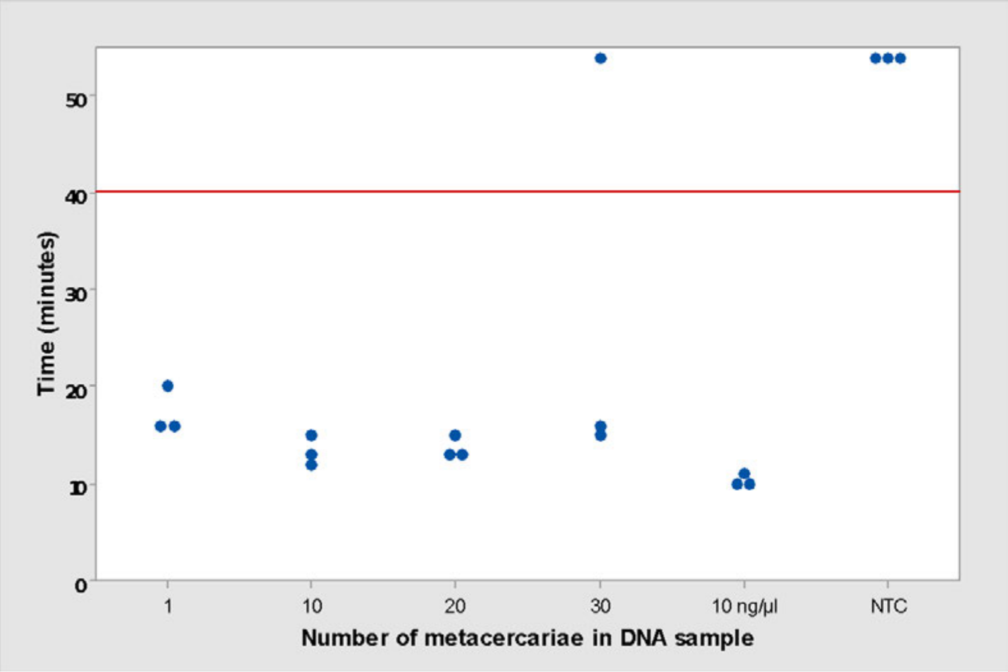


Figure 5.9 - Individual dot plot representing LAMP amplification of *F. hepatica* COX1 target from DNA extracted from different numbers of metacercariae using time to detection

The ability of LAMP to detect a single metacercariae from grass is shown in Figure 5.10. No samples had all three replicates positive for the *F. hepatica* COX1 target. Positive replicates were all detected after twenty minutes. Samples F and G were not positive for the target (Figure 5.10). The target was amplified from all replicates of the positive control before ten minutes and no amplification from any of no target control replicates.

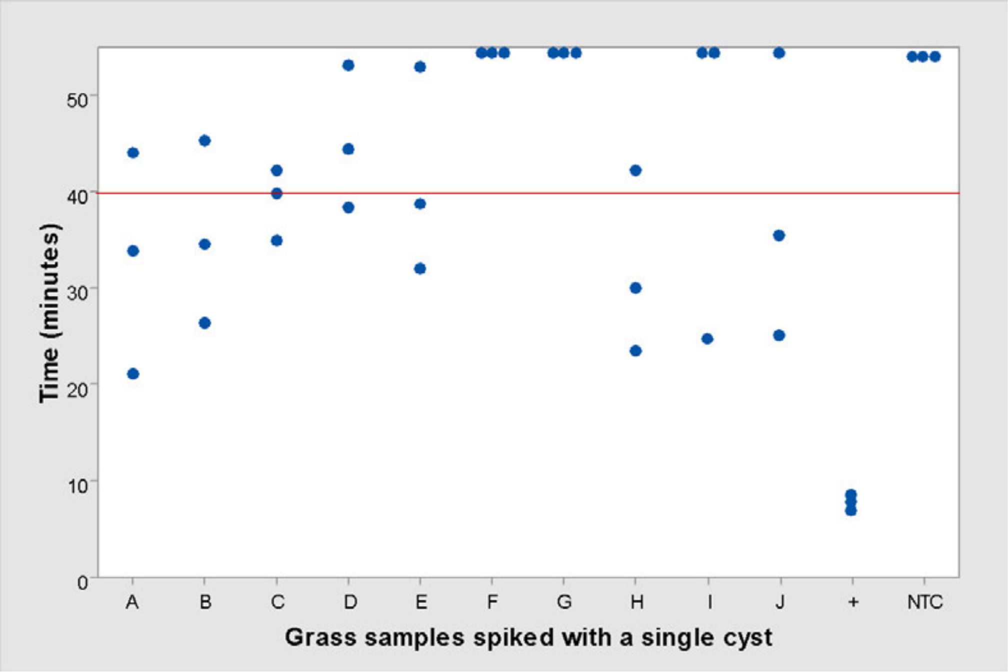


Figure 5.10 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from samples A-J, tested in triplicate. A-J = individual 0.05g grass samples spiked with a single *F. hepatica* cyst prior to DNA extraction + = Positive control, 10ng/µl *F. hepatica* DNA, NTC = NF H_2O

Twelve subsamples taken from grass spiked with one hundred metacercariae were tested using LAMP. Four samples were positive for the *F. hepatica* COX1 target (A, G, I and K) (Figure 5.11). Detection of the target appears on the forty minute cut-off line for samples C and D. The results for C, D and the one replicate of the no target control are considered to be false positives.

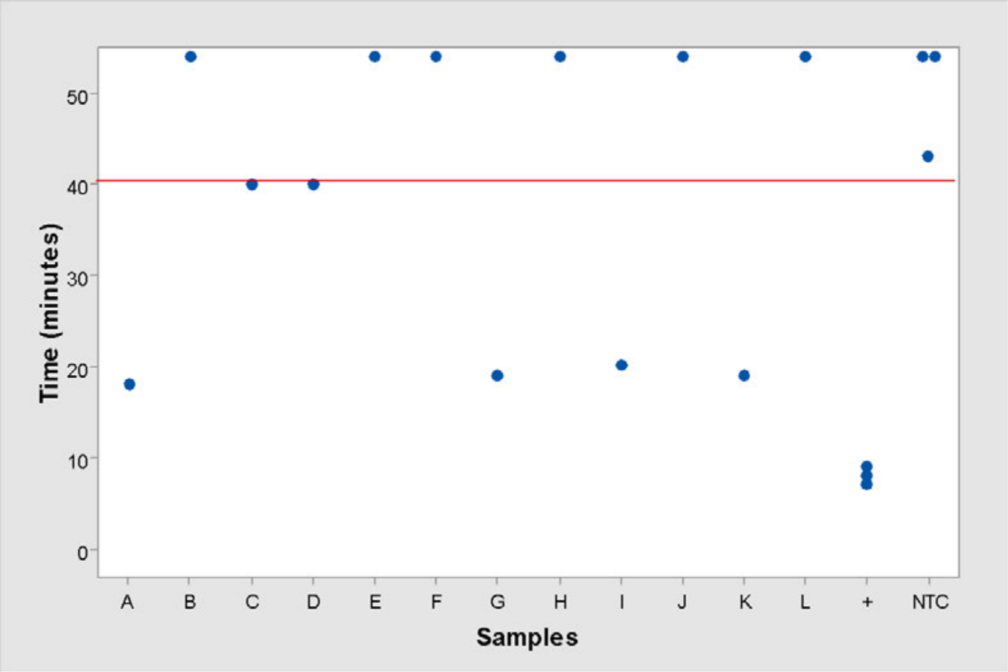


Figure 5.11 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from samples A-L, tested in triplicate. A-L = DNA extracted from 0.05 g subsamples of grass spiked with 100 cysts. + = 10ng/µl *F. hepatica* DNA and NTC = NF H_2O

In order to determine the limit of detection of LAMP assays were performed on *F. hepatica* DNA serial diluted in water and in DNA extracted from grass. The target was identified in DNA concentrations 1, 0.1 and 0.01 ng/µl in all three replicates before the forty minute cut-off. Two out of three replicates were positive for the target at DNA concentrations 0.001 and $1x10^{-4}$ ng/µl. The target was not detected from DNA samples concentrations $1x10^{-5}$ ng/µl or $1x10^{-6}$ ng/µl. A trend in the data suggests that as the concentration of *F. hepatica* DNA increases, the time to detection decreases. The detection of the target from the positive control replicates clustered around ten minutes. False amplification occurred in seven out of ten grass no target controls (Figure 5.12).

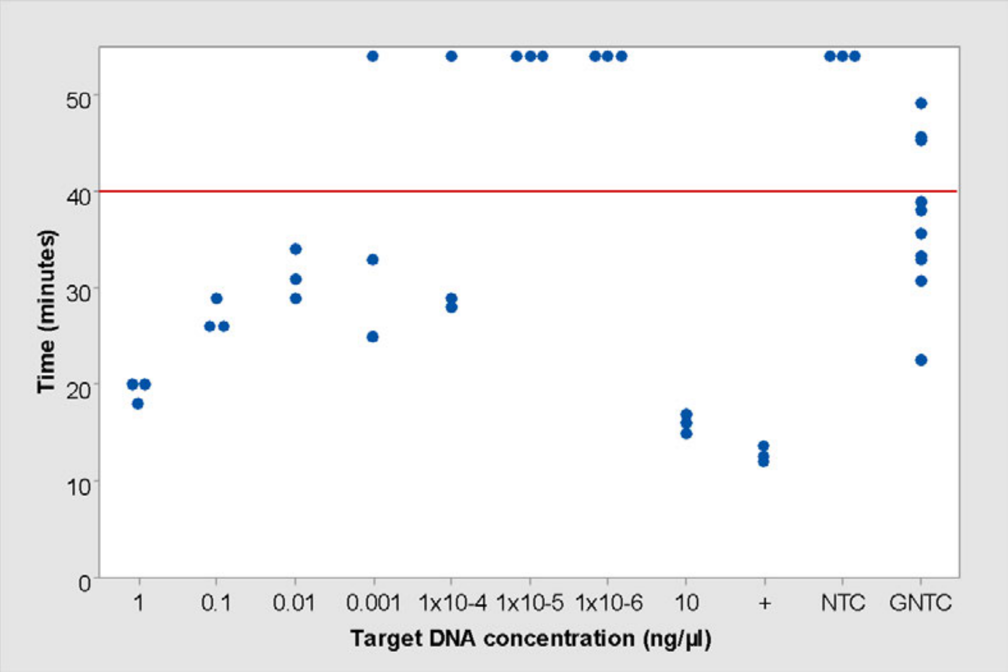


Figure 5.12 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in water, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H_2O. GNTC = 10 separate grass negative controls; DNA extracted from grass

LAMP successfully detected the *F. hepatica* COX1 target from all three replicates of DNA concentrations 1, 0.1, 0.01 and 0.001 diluted in DNA extracted from grass. Two replicates of DNA concentration 1x10^{-4} ng/µl were positive for the target. False amplification resulted from two replicates of DNA concentration 1x10^{-5} ng/µl. This assay was performed on the same plate as the assay depicted in Figure 5.12, both assays share the same positive and negative controls (Figure 5.13).

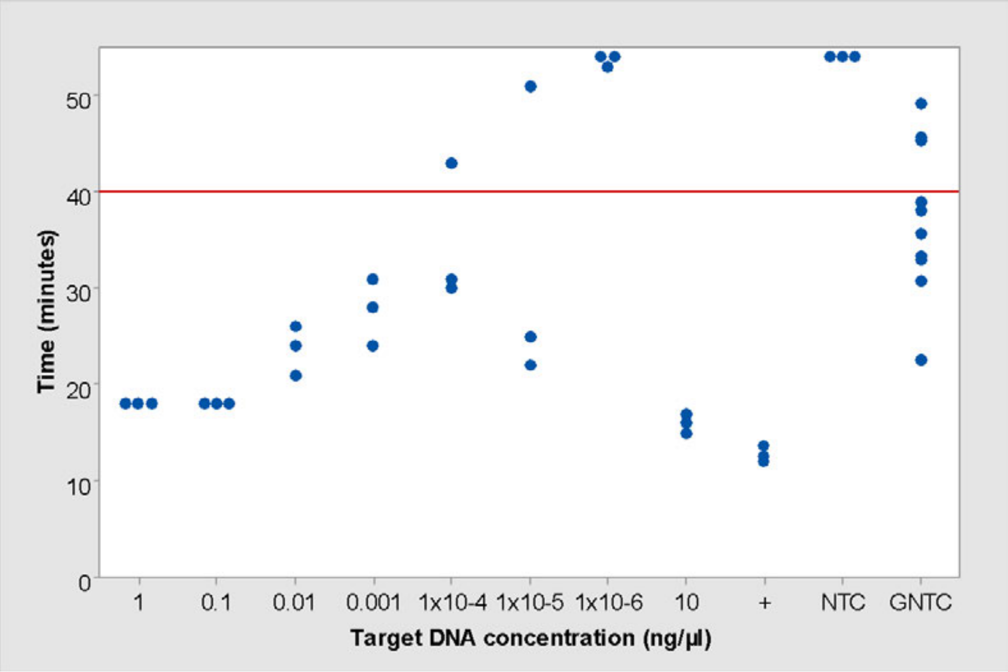


Figure 5.13 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in DNA extracted from grass, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H_2O. GNTC = 10 separate grass negative controls; DNA extracted from grass

The assay results displayed in Figures 14 and 15 depict the limit of detection of LAMP assays performed on *F. hepatica* DNA serially diluted in water and in DNA extracted from grass. These assays included less Tris buffer in the reaction mix than the previous LAMP assays. The limit of detection of LAMP under these conditions is 0.01 ng/µl when *F. hepatica* DNA is serially diluted in water (Figure 5.14). The target is detected in two replicates of DNA concentration 0.001 ng/µl and one replicate of 1x10^{-6} ng/µl diluted in DNA extracted from grass (Figure 5.15). Again both assays were performed on the same plate utilising the same controls. Detection of replicates of 10 ng/µl cluster around fifteen minutes and the synthetic control around ten minutes. False amplification is not observed in any of the grass no target controls (Figure 5.14 & 5.15).

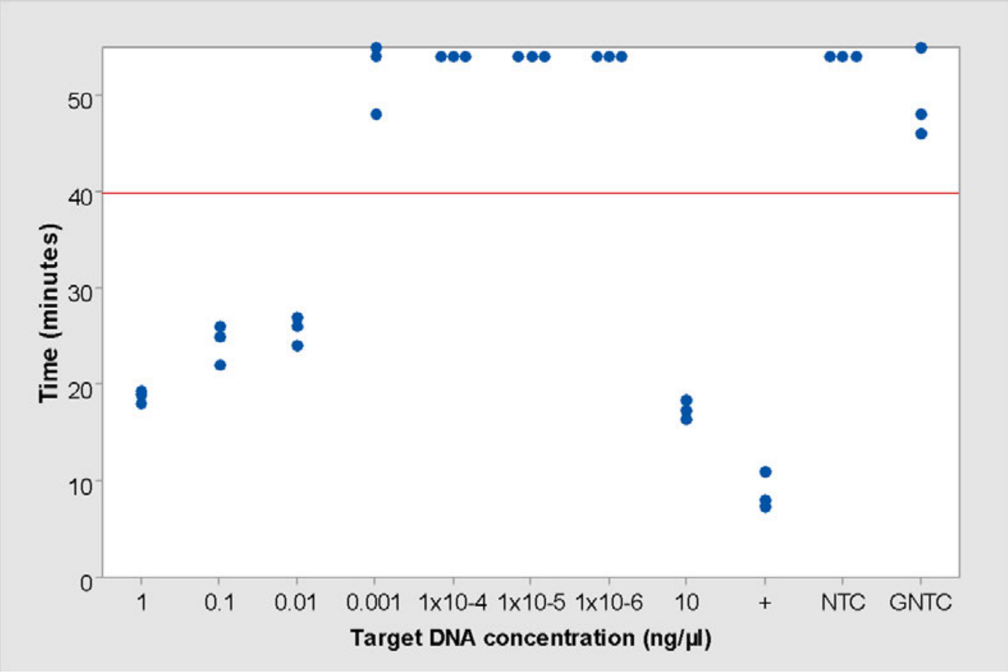


Figure 5.14 – Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in water, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. Each dilution was run in triplicate. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H2O. GNTC = Grass negative control; DNA extracted from grass

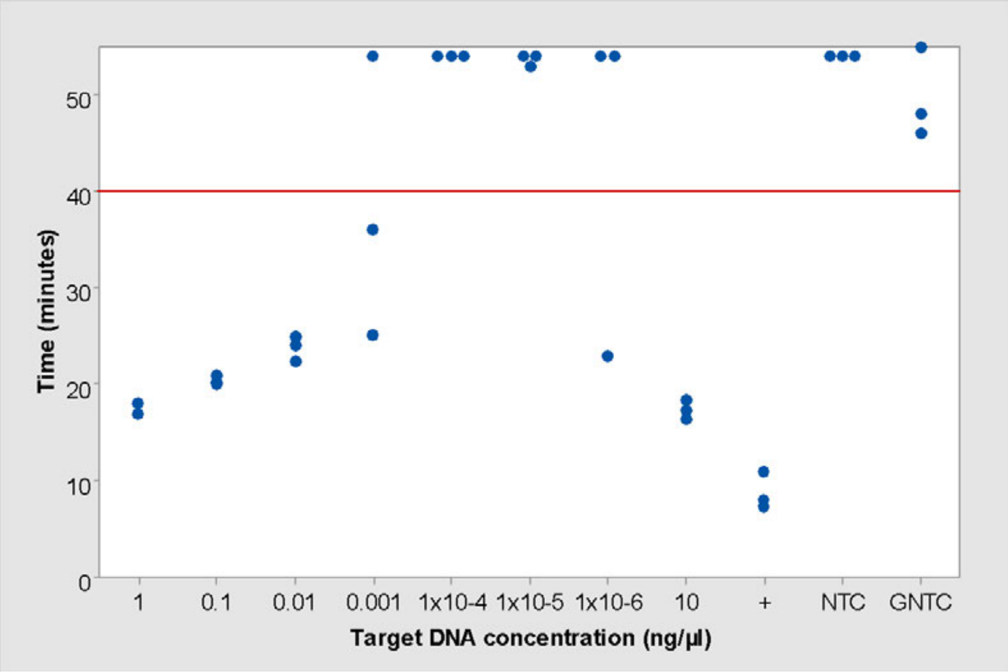


Figure 5.15 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in DNA extracted from grass, and from 10ng/µl of

F. hepatica DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H_2O. GNTC = Grass negative control; DNA extracted from grass

The same assay design and conditions as performed for Figures 5.4 and 5.15, including the same concentrations of Tris buffer, were repeated. Both assays depicted in Figures 5.16 and 5.17 were performed on the same plate utilising the same controls. The results are displayed in Figures 5.16 and 5.17. The target was detected around fifteen minutes from the synthetic positive control. Detection of the target occurred between twenty and thirty minutes from DNA concentrations 1, 0.1, 0.01, 0.001 and 1x10^{-6} ng/µl when *F. hepatica* DNA was diluted in water (Figure 5.16).

The target was detected in DNA concentrations 1, 0.1, 0.01, 0.001 and 1x10 $^{-4}$ when *F. hepatica* DNA is diluted in DNA extracted from grass. Detection of the target started at around seventeen minutes and ended at around thirty three minutes (Figure 5.17). False amplification was not observed in any of the grass no target controls (Figures 5.16 & 5.17).

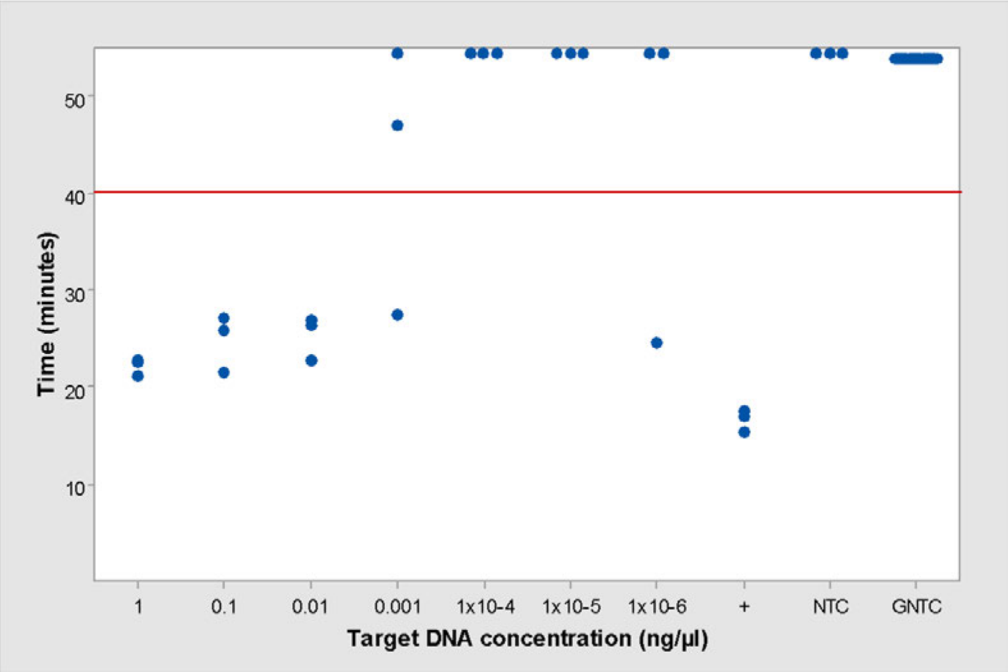


Figure 5.16 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in water, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. Each dilution was run in triplicate. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H2O. GNTC = 10 separate grass negative controls; DNA extracted from grass

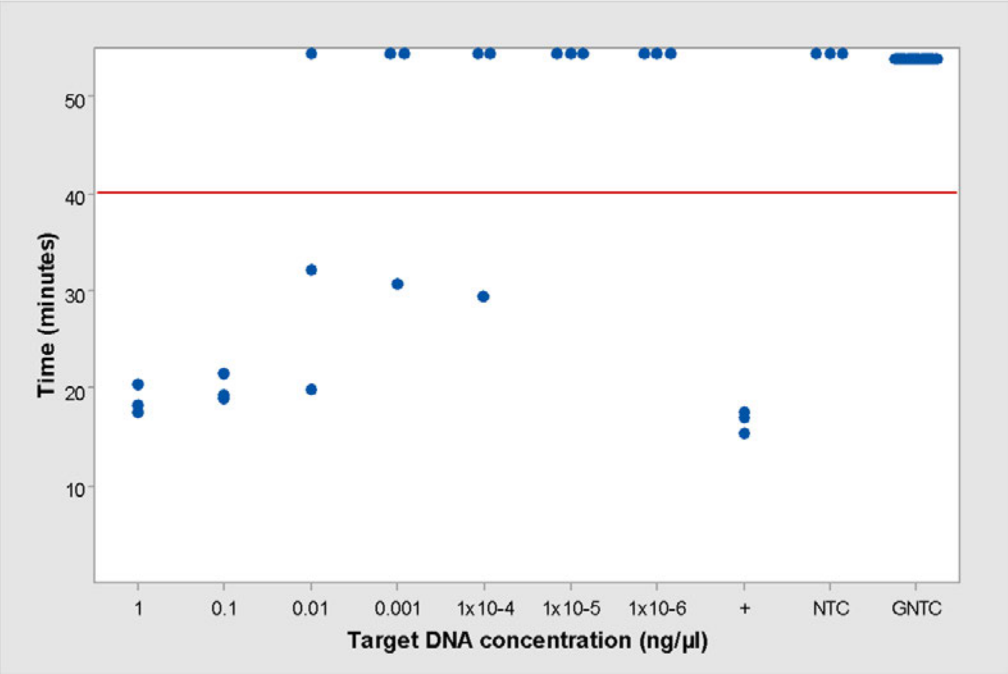


Figure 5.17 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in DNA extracted from grass, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H_2O. GNTC = 10 separate grass negative control; DNA extracted from grass

5.4 Discussion

This Chapter aimed to (1) demonstrate the use of both PCR and LAMP for the detection of the *F. hepatica* COX1 target from samples of grass DNA spiked with *F. hepatica* DNA and from grass spiked with metacercariae and to (2) evaluate the possible application and translation of these tools for forecasting fasciolosis on farms and in the environment.

5.4.1 Performance of PCR and LAMP

Overall, the results of the work carried out for this Chapter show that it is possible to detect *F. hepatica* metacercariae from spiked grass samples using PCR and LAMP. Two sample scenarios were used to demonstrate this proof-of concept study. One sample scenario used serial dilutions of metacercarial DNA in DNA extracted from grass. This was done in order to understand the limit of detection of both PCR and LAMP when amplifying the COX1 target in the presence of grass DNA and products of its DNA extraction. In the second scenario fresh grass was spiked with metacercariae prior to DNA extraction. These samples represented pasture samples and were used in order to mimic "real farm" samples as a way of understanding how PCR and LAMP might perform on "real farm" samples but in a controlled way.

The amplification of the *F. hepatica* COX1 sequence was successfully demonstrated using PCR and LAMP in both scenarios with varied results. Both methods of DNA amplification have the capability to identify very small concentrations of *F. hepatica* DNA, with detection sensitivity strong enough to detect DNA below the expected concentration of DNA from a single cyst (~2 ng/µl). The variation in results centred on the inclusion of grass in samples. PCR had issues with sensitivity and LAMP had issues with false amplification when grass DNA and its extracts were included.

LAMP performed comparatively better than PCR in the presence of grass and its extracts. The limit of detection of LAMP when metacercarial DNA was diluted in DNA extracted from grass was 100 times lower than PCR (0.001ng/µl vs 0.1ng/µl). At the limit of its detection LAMP could amplify the COX1 target in under 30 minutes. By altering the concentration of Tris buffer included in the LAMP assay, it was possible to remove any false amplification caused by the inclusion of grass with minimal effect on the limit of detection. False positives are a commonly associated problem with LAMP because of the use of several primer couples and the isothermic nature of amplification (Wang et al., 2015). False positive signals could have resulted from non-specific interactions and/or primer dimers. By decreasing the concentration of Tris buffer used in the LAMP reactions the appearance of false positives from samples including grass extracts was removed.

The initial 40-minute cut off time was determined from data collected by MAST using a synthetic positive control and their own in-house LAMP primer set. This kit was designed to detect a wide range of varying targets using a 'catch-all' master mix which the user can add their own LAMP primer set to. This means that the cut-off time suggested is a guide. Many of the positive samples amplified using a master mix containing less Tris buffer than the MAST guidelines suggest appear before 35 minutes, most before 30 minutes. Replicate concentrations 1, 0.1 and 0.01 ng/µl appear in clusters before 30 minutes. Changing the cut-off time to 30 minutes would alter the limit of detection to 0.01 ng/µl in both assay scenarios. This would not impact the detection of *F. hepatica* COX1 as this concentration of DNA would not equate to high level of liver fluke risk.

PCR primers used to detect the *F. hepatica* COX1 target did not amplify DNA extracted from grass. This primer set (Martìnez-Pèrez et al., 2012) has already been shown to have high specificity to *F. hepatica* and there is no interaction with other trematodes commonly found in the same environment as *F. hepatica* (Appendix 4). Despite the high specificity of this PCR assay, sensitivity was hampered by the inclusion of grass and its extracts. The limit of detection of PCR when metacercarial DNA was diluted in water was 1×10^{-4} ng/µl but this was reduced to 0.1ng/µl when metacercarial DNA was diluted in DNA extracted from grass.

The reduced sensitivity of PCR when grass and its extracts are present in a sample suggest that the PCR assay is somehow inhibited. Inhibition could have been caused by substances released during DNA extraction of grass. Plant polysaccharides are commonly associated with inhibition of PCR assays when amplifying targets from plant material (Wilson, 1997). The mode of inhibition polysaccharides play in PCR is not fully understood (Demeke & Adams, 1992) but it is believed that they reduce the efficiency of the *Taq* polymerase by being structurally very similar to nucleic acid thus binding to the enzyme and reducing DNA amplification (Schrader et al., 2012). Other common inhibitory substances associated with plant material and environmental samples are humic compounds. These are capable of causing PCR inhibition either by binding to the DNA template, thus preventing binding of the primers, or by disrupting the activity of the DNA polymerase (Matheson et al., 2010).

The reduced level of sensitivity of PCR in this scenario would appear to be inconsequential when considered in the context of developing a presence/absence based test and the potential translation of this test into a tool for prediction of metacercarial challenge. A single metacercaria does not in itself equate to a high level of infective risk and one metacercaria can yield ~ 2ng/µl (personal observation) of DNA which is well within the limit of detection for PCR.

Key to identification of *F. hepatica* DNA from the environment is the ability to amplify DNA from pasture samples which, most commonly, are complex, full of detritus, and potential inhibitors. LAMP is the most suitable choice to take forward as a tool for the detection of metacercariae from pasture and as an ‘on farm or ‘point of care’ test.

5.4.2 Application and translation

Issues with LAMP

High sensitivity in an assay is usually a desirable trait, however in the case of LAMP it can be cumbersome. Great care must be taken while setting up an assay to avoid contamination resulting in false positives. LAMP amplifies the DNA target into thousands of copies. Opening tubes or plates after a reaction is a cause for concern in terms of contamination of workspaces, especially if working in a lab space where different groups work on the same species. LAMP is also vulnerable to carry-over contamination whist setting up an assay (Kila et al. 2015). The occurrence of amplification in LAMP assay past the limit of detection is mostly likely the result of aerosol contamination during set up of the experiment. The contamination is likely to have occurred during the pipette dispensing of the dilution series of metacercarial DNA. The proximity of the wells on a 96 well plate may well have aided in the accidental dispersal of extra DNA into the wrong well. Applying commonly used practices of biosafety, such as wearing gloves, working under isolated UV hoods and using new pipette supplies with every experiment are good laboratory practices to adhere to in order to avoid cross contamination (Kwok & Higuchi, 1989).

No reactions were opened after amplification, but this also meant that LAMP products were not sequenced for validation. PCR product can be sequenced, this was used to validate the identification of single cysts from grass using LAMP.

5.4.3 Further work and considerations for an on farm test

The results of the PCR and LAMP assays performed in this chapter highly suggest that the same methods could detect metacercarial DNA from ‘real farm’ samples. Despite this, there exist a number of barriers and considerations which need to be addressed before either test could be translated for use with pasture samples or as a ‘on farm’ test.

Other life stages

The most likely source of environmental DNA (eDNA) from *F. hepatica* is from miracidia, the tails of cercariae and from dead cercariae (Jones et al., 2018). It was argued that it is less likely that any eDNA would be originating from metacercariae unless the metacercariae were dead or damaged. The double cyst wall is thought to prevent any leakage of DNA into the environment (Jones et al., 2018). However, it should be noted again that the worked performed by Jones et al., (2018) was on water samples. It is likely that the concentrations of DNA from each life stage would be reversed with pasture samples. The highest concentrations of *F. hepatica* DNA in pasture samples would most likely be from eggs and metacercariae as these life stages are designed to remain viable on the pasture for longer periods than miracidia and cercariae. Additionally, although water is important for the survival of eggs and metacercariae on pasture, it is not a direct requirement of their purpose. Miracidia and cercariae require water in order to complete their purpose in the life cycle. Miracidia travel through water in search of a snail host and cercariae use water to find place to encyst so it is logical that their DNA would be in the highest abundance there.

Eggs, miracidia and cercariae are all life stages of *F. hepatica* that could be present on the pasture along with metacercariae. It is not possible to determine what life stage the COX1 target was amplified from the results of a PCR or LAMP assay. The DNA of *F. hepatica* does not change throughout its lifecycle but certain genes are expressed at different levels depending on the life stage. The expression of a tegument associated protein, FhTP16.5, was investigated in different *F. hepatica* life stages using quantitative reverse transcriptase PCR (RT-qPCR) (Gaudier et al., 2012). The analysis showed that there is no expression of FhTP16.5 in eggs, very little expression in miracidium and high expression in juveniles and adult stage of *F. hepatica*. No data was collected for cercariae or metacercariae. The differences in expression of this protein were explained to be caused by the life stage. A lack of need for this protein during the formation of the eggs was proposed as the reason behind the lack of expression of FhTP16.5 in eggs. The low expression of FhTP16.5 within the ciliated epithelium of miracidium was suggested to act as nutrition for the larvae as its searches for and penetrates *G. truncatula*. High levels of expression within the juveniles and adult was proposed to support the theory that the FhTP16.5 protein is important in the survival of these stages when in the definitive host. Investigating the expression of FhTP16.5

protein in metacercariae may offer a possible method to differentiate metacercariae from other life stages in a pasture sample.

Viability and Quantification

Although an RNA based test has the potential to determine the origin of a DNA target from a pasture sample the practical application of this would not be realistic. The results of either an RNA or DNA based would not be powerful enough just on their own, especially in the context developing tests for disease forecasting. The recovery of metacercariae coupled with the results of a LAMP assay would offer much more valuable information. The manual recovery of metacercariae from pasture allows extra epidemiological information to be collected, namely, the quantity and viability of metacercariae on the pasture. Recovered metacercariae can be counted and then excysted to determine the viable risk to grazing livestock. This can be applied to pasture samples that were positively identified from a LAMP assay. Or alternatively the species identity of any metacercariae can be confirmed by DNA assay after counting and excysting them.

Timings, presence and evaluation of risk

Presence of fluke DNA within a sample does not always correlate with the presence of a complete working lifecycle with live life stages in the sample area. The species may have been present in the past and is now no longer present because of insufficient conditions for the life cycle to complete, namely the absence of a host species in the environment. Time of year, weather and the presence of intermediate host species are other important factors when considering the risk of fluke on farms. Performing other diagnostics tests, such as faecal egg counts, snail surveys and the recovery of metacercariae from pasture would confirm a viable presence of *F. hepatica* on a farm or field.

It is important to consider the strength of a positive result in relation to the risk of fasciolosis. For example, one positive sample or replicate from an assay performed on many samples taken from the same field is not a strong indication of high fluke risk. DNA detection can be most successfully applied through a combined approach. The level of risk must be considered on the basis of all available evidence. DNA detection can be used in the first instance to pinpoint areas of fields where liver fluke is present or as a confirmation of species identity after sample processing. But it is important to utilise all diagnostic tools at hand when deciding the level of fasciolosis risk. Considering the history of fasciolosis on a farm, assessing weather readings, performing snail surveys and manual recovery of metacercariae from pasture offers more comprehensive understanding of fluke risk on a farm. Combining data from many different farms around the country allows for the potential for greater epidemiological understanding of when and where the highest fluke risks might occur, thus leading to more accurate and reliable fasciolosis forecasts.

DNA extraction

The incorporation of larger sample sizes must be considered for the potential application of LAMP testing of pasture on farms. Optimal DNA extraction is key to DNA identification tests. Improper breakdown of material will result in the target of interest not being available for amplification which then can lead to false negative results. The failure to detect the target from samples of grass containing a single cyst may indicate that DNA extraction using this method is not optimal. Two out of ten grass samples spiked with a single cyst were not positive for the target when amplified with LAMP. Despite the tiny amounts of grass used in each staged sample this may have been too much material to ensure complete breakdown of the metacercariae during DNA extraction. Single cysts may have been shielded from enzymatic breakdown by the grass therefore the COX1 target would not have been made available for amplification.

It is particularly challenging and expensive to extract high quality DNA from environmental samples using DNA extraction kits, because of the plant material present (Inglis , Pappas, Resende & Grattapaglia, 2018). The DNeasy (Qiagen) kit used in this study is expensive and not suited for large scale DNA extraction on plant material. The largest sample of grass from this study was 1.5g of grass. Although appearing to be a small sample size, in practice this volume of grass required blending and then subsampling before this could be put into Eppendorfs for DNA extraction. A more suitable method of isolating the *F. hepatica* COX1 target DNA extraction from pasture samples may include the use of magnetic beads (Hawkins, 1998) or the use of affinity purification (Kadonaga & Tjia, 1986). However, even these methods might be too costly to be considered as a ‘on farm’ inexpensive test.

In order to test a farm for the presence of *F. hepatica* using LAMP, many samples of grass from one field would be required. The protocol of field sampling for pasture sample for DNA analysis might follow similar methods as those for the recovery of metacercariae. One method for the recovery of rumen fluke metacercariae describes sampling grass in a ‘W’ format in fields at over 200 locations (O’Shaughnessy et al., 2018). This method is based on the recovery of metacercariae from herbage which suggests collection of 100g of grass per individual sample (Ministry of Agriculture, 1986) This is 100 times more grass than the largest sample used in this study. Performing DNA analysis on sample sizes this big would be impractical. Alternative methods of sample processing and DNA extraction that could handle larger samples sizes efficiently and at low cost would be the ideal solution.

5.5 Conclusion

The experiments performed in this Chapter have demonstrated that it is possible to detect very low concentrations of metacercarial DNA and numbers of metacercariae from grass that has been purposely spiked. PCR and LAMP both demonstrate capability of detecting the *F. hepatica* COX1 target from DNA samples containing grass DNA and other components of the extraction process. These results are promising when considering the highly complex nature of DNA samples collected from a field situation. Despite this, the application of these methods in a farm situation may be unsustainable. Without proven identification of metacercariae on the pasture by isolation and recovery any results of a DNA test from grass would be meaningless for an epidemiological review. There also exist many practical barriers for the 'on farm' application of a DNA assay. The issue of sample size, DNA extraction and making the assay end point user friendly are the main challenges facing the translation of a LAMP assay for use on farm.

After consideration of the results, it is recommended that DNA testing be used as a way of determining species ID of metacercariae recovered from pasture or as a quick, but crude pin point determination of where fluke has and could be present on the farm. The recovery of metacercariae is a much more logical method of determining fasciolosis risk on pasture as the results can give much more information as to the numbers and viability of metacercariae on pasture.

Chapter 6: Discussion

6.1 Determining metacercarial viability

Light microscopy and excystment were considered to be the most appropriate methods to investigate and compare for this project. Both these methods fit the criteria for the development of tests which can contribute to the rapid determination of *F. hepatica* risk on pasture.

The results of Chapter 2 highlighted that morphological assessment of metacercariae, using light microscopy, is not a reliable method to determine metacercarial viability. Metacercariae can appear morphologically sound and show no signs of deterioration but the juvenile fluke will fail to excyst. Obviously damaged and or decaying metacercariae are easier to identify using light microscopy and their viability status is predictably non-viable when processed through an excystment assay. Despite the inaccuracy of using visual assessment of metacercarial morphology as an indicator of viability it is still useful and necessary to use light microscopy when maintaining populations metacercariae under lab conditions. Laboratory technicians require light microscopy when handling metacercariae for experiment procedures. Excystment assays require light microscopy too observe the excystment of juvenile flukes.

The C. E. Bennett method of excystment was an inappropriate method of measuring metacercarial viability because the conditions of the assay did not meet the requirements to stimulate excystment. The modified Hernández-González et al., (2010) excystment assay was demonstrated effectively with both ovine and bovine bile. It was used successfully in this thesis to determine the effect of pH and ensilaged on metacercarial viability. Despite the success of this assay there exist some barriers to the translation of its use to help forecast metacercarial challenge on pasture. The establishment of on farm or in the field excystment assays may be possible but there are still many barriers to their application. Key equipment for this test are microscopes, Petri dishes and incubators, these are not readily found on farms and are expensive to buy and maintain. Reagents; sodium dithionite, HEPES and Hanks' balanced salt solution are only supplied by scientific companies and are expensive to purchase. Bile can be sourced from dead animals but again it is not readily available. Using carbonated water in this test is the most user friendly aspect as this is readily available and easier to store than canisters of CO_2. Although, relatively easy to perform, training is required to understand the different conditions of the assay and what a viable and non-viable metacercaria looks like.

Key to the rapid determination of fasciolosis risk is the use of quick reliable methods. The passage of metacercariae through animals to get results on infectivity can take weeks. There are also costs involved with post mortem and recovery of adults fluke from the liver. Excystment assays can provide results within hours compared to weeks. There are also many other advantages to using excystment assays. Excystment assays are advantageous over the use of animal models as the newly excystment flukes can be collected and used in further research such as identification of drug and vaccine candidates (Garcia-Campos, Baird, & Mulcahy, 2016; Hernández-González et al., 2010). The speed of results and ease of performing excystment assays are advantageous that are most applicable to the topic of this thesis. Rapid results are important to farmers as preventable action can be implemented more effectively if time is available to arrange alternative low risk grazing or to manage high risk areas on pasture by performing drainage or fencing off these areas.

In a study conducted on *F. gigantica* metacercariae which investigated metacercarial viability at different temperatures and humidity, dead metacercariae were described as having 'visible necrosis' (Roberts and Copeman, 2006). This study included extra investigatory steps to accurately determine metacercarial viability. Microscopically this study categorised the viability of the newly excysted flukes as 'live' (motile and undamaged) and dubious (not motile but undamaged) and then infected groups of sheep separately with the two different categories of fluke. The percentages of adult flukes recovered from each type mirrored their previously assigned viability category. 54% of ‘live’ newly excysted fluke were counted as adults compared to 7% of ‘dubious’ fluke (Roberts & Copeman, 2006). The percentages of adult flukes recovered from both the ‘live’ dubious groups highlights that although a metacercaria may excyst the juvenile fluke may not establish an infection.

The results of these assays can only determine if a metacercariae can excyst, they cannot predict if the excysted fluke will establish as adults causing a parasitic infection. Infectivity of metacercariae is perhaps much more important and relevant to the topic of forecasting fasciolosis. Infectivity is closely associated with severity of infection. If many infectious metacercariae are consumed at once or over a short period this can lead to acute disease and possibly death, in sheep. Chronic disease in sheep and cattle, is associated with the 'trickle' effect which is the ingestion of small numbers of viable infectious cysts over a long period of time (Behn and Sangster, 1999).

There have been studies that have used animals such as unexposed 'tracer' sheep and guinea pigs to determine the fluke risk on pasture (Olsen, 1947; Ross & O’Hagan, 1966). Ross and O’Hagan fed guinea pigs grass from areas of pasture where infected snails were located. From their own previous investigations they knew the infectivity of these metacercariae was around 33.33%. From one guinea pig fed 6oz of grass, 3 fluke were recovered. They were able to estimate the number of metacercariae per lb of grass by multiplying the number of fluke recovered, 3, by 2.666 (corrected from oz to lb, 1lb =16oz, 16oz ÷ 6oz = 2.666), then dividing by the arbitrary infectivity of 33.33% (3*2.666) ÷ 0.3333 = 24 metacercariae per lb of grass) (Ross & O’Hagan, 1966). The use of lab animals is not conducive to the quick assessment of metacercarial viability. Their use might be important in development infectivity prediction models but excystment assays should be used where farmers require a quick assessment of fasciolosis on pasture.

Determining metacercarial viability is key to a better understanding of liver fluke epidemiology. The development of new forecasting models requires data on the percentages of viable metacercariae on pasture, particularly from wild populations of metacercariae. From the work performed in this thesis it has been highlighted on that the 'on-farm' translation of excystment assays is not practically feasible because of the equipment and reagents required. What may be more achievable and advantageous to fluke forecasting is the creation of surveillance labs around the country. These labs could receive grass samples and from the metacercariae recovered determine their viability. This data could be combined with previous year’s prevalence data, the history of fasciolosis on farms, the observance of snail habitats, meteorological data, numbers of metacercariae recovered from pasture and their excystment percentage. This combined data could be used to generate probability scores predicting the severity of infections and be included in new forecasting models.

6.2 Recovery of metacercariae from grass

Importantly, for a surveillance scheme to be implemented a reliable effective method of metacercarial recovery form pasture must be determined first.

It was possible to recover metacercariae from purposefully spiked grass and cellophane using vinegar and a salad spinner. This method was also somewhat successful when performed on grass infected with *Notocotylus sp.* metacercariae. Cellophane rafts were not successful in recovering *F. hepatica* metacercariae from wader scrapes. Although, it is presumed that infected snails were not in high enough abundance to shed detectable numbers of metacercariae or to shed metacercariae into the water.

The development of a reliable effective method for the recovery and isolation of metacercariae from pasture is challenging. As demonstrated in this thesis, even when grass is spiked purposefully with metacercariae is still difficult to recover them again. Without the methods to gather information as to the numbers and viability of metacercariae on pasture the accuracy of fasciolosis models cannot be improved upon.

The use of vinegar and a salad spinner to recover metacercariae from pasture was inspired by previous publications that used a combination of chemical and mechanical methods to recovery metacercariae (MAFF 1986; El-Sayad et al.,1997 and O'Shaughnessy et al., 2018). These methods were successful when performed upon grass and salad samples. However, without knowledge of the numbers of metacercariae encysted in the samples no comment can be made as to their efficacy. Future development of metacercarial recovery methods and or assessment of the efficacy of different recovery methods must include the use of infected *G. truncatula* colonies to shed onto laboratory grown grass. These samples are most representative of infected pasture samples and also allow for the controlled determination of method efficacy. The number of metacercariae present in any mocked grass sample can be derived from knowledge of snail cercarial shedding rates, the number of snails present and time of snail exposure to the grass. A calculation using these known facts can provide a close estimation of the number of metacercariae presence in a mocked grass sample.

Further exploration of metacercarial recovery methods might take the approach of selecting particular aspects of the El-Sayad et al., (1997), O'Shaughnessy et al., (2018) and Fry, et al., (1984, 1985) methods of recovery and combining them. The use of vinegar (El-Sayad et al., (1997) could be used in the fist instance to detach metacercariae from grass. This grass and vinegar mixture could then be vigorously agitated in big buckets by machinery instead of by hand (O'Shaughnessy et al., 2018). The use of a salad spinner (El-Sayad et al., 1997) to separate grass from metacercariae could be scaled up and included in to the big buckets so the metacercariae are recovered during agitation and washing. The remaining solution containing recovered metacercariae could then be separated via density gradient using

Percoll and Metrizamide, similarly to Fry, et al., (1984, 1985), and centrifugal force to separate and isolate viable and non-viable metacercariae.

A method in which to survey and sample fields for metacercariae in a representative way is required. MAFF (1986) suggests a random sampling procedure taking no more than 100g of fresh grass per sample and cutting the grass as close to the base as possible without including soil. Sampling procedure could follow the ‘W’ approach (O’Shaughnessy et al., 2018), but include sampling of the same fields or areas multiple times throughout the liver fluke season (May-October) or year. Thus providing representative data of metacercarial presence and viability over time.

6.3 Survival of metacercariae in grass silage

pH associated with different qualities of silage fermentation was determined not to be a significant factor of *F. hepatica* metacercarial viability. Metacercariae survived in lactic acid solutions at pHs 4, 5 and 6 for 4 weeks. At 16 weeks at pH 4, 38% of metacercariae were still viable. In laboratory spiked silage samples of 'high' quality, 14 or 24 recovered metacercariae survived 4 weeks. From this is was determined that pH is not a determining factor of metacercarial survival in silage. What the results did highlight was that recovery of metacercariae from grass silage is difficult without an effective recovery method.

The results of the experiments performed in Chapter 4 are effected by the metacercarial recovery rate from the silages. Similarly to fresh grass, silage is a difficult substrate to work with. The recovery method used was unreliable and future assessment of the effect of silage fermentation of metacercarial viability would benefit from more robust and reliable recovery method. The method approach explained in section 6.2 should suit both fresh and ensiled grass.

The experimental design did not allow for the determination of metacercarial survival throughout the grass ensilage process. Metacercariae were only introduced to the silage after the initial fermentation phase was complete and the conditions of the silage was stable. The most dramatic changes to the conditions of the ensiled grass are during the first four stages of fermentation (Seglar, 2003). Most related to the survival of metacercariae is the temperature change associated with the activity of lactic acid bacteria during this initial phase. Future assessment of this effect of this stage on fermentation on metacercarial survival would require a colony of *F. hepatica* infect *G. truncatula* to shed on to grass for ensilage. The full effect of the ensilaging process on metacercarial viability could be assessed under laboratory conditions. A time course experiment could be designed allowing for the opening up individual samples of ensiled grass at different timepoints and recovering the metacercariae.

Another important factor in metacercarial survival is the presence of adequate moisture to defend against metacercarial desiccation. However, metacercariae were able to survive 4 weeks in silage with an average dry matter content of 321.5 g/kg. This was the highest DM content of the three silage qualities but the survival of metacercariae in the 'medium' and 'low' quality silages were not tested due to metacercarial supply. Silage quality was assessed from data gathered by Near Infra-Red Spectrometry (NIR). This is commonly used by labs to predict the quality of silage. A more accurate assessment of Dry Matter (DM) content and other silage components would have dried the samples at 100°C and performed wet chemistry to assess other feed components.

It is possible for *G. truncatula* to have suitable habitat on silage fields. The lifecycle of *F. hepatica* can be maintained on these fields even without livestock grazing them. Slurry containing *F. hepatica* eggs can be spread on to these areas and the lifecycle continued from the result of hatched miracidia finding *G. truncatula.* This can be avoided, however if the slurry is heat treated prior to application (Kelley et al., 2016). Correctly treating stock with anthelmintics will also reduce the presence of eggs in faeces.

It is generally accepted that if farmers follow the standard practice for silage production and avoid the common causes of silage spoilage then there is very little risk of *F. hepatica* presence in silage. The effect of anaerobic spoilage on metacercarial survival has been highlighted as an additional research priority (John et al*.*, 2019).

6.4 DNA detection of *F. hepatica* metacercariae on pasture

DNA testing could remove the need to perform the difficult and often time-consuming tasks of snail hunting and/or herbage examinations for metacercariae. Snail 'hunters' need to be skilled in the identification of this small mud snail. An arising issue with the observance of snails in the environment is that the known habitats are expanding and changing with the effects of climate change (Kenyon et al., 2009). This means that *G. truncatula* is operating in habitats that are currently unknown, resulting in uncharacterised areas of high fluke risk.

The success of snail hunting depends on a number of variables. Climatic conditions of both the day of sampling and on the days leading up to that can influence the number of active snails on the surface. Below 7˚C snails are not active and will cease any activity below 1.5˚C (Kendall, 1953; Richards, 2016). Snails may burrow in to the soil when temperatures are not favourable making them difficult to spot (Dreyfuss, Vignoles, & Rondelaud, 2015). Favourable temperatures for snail growth and activity are often referenced to be within the range of 16-22˚C (Kendall, 1953; Nice, 1979; Richards, 2016).

There is very little published data on the numbers and distribution of metacercariae on plants. Any data that is published was collected over many years. A study of metacercarial load on 247 beds of watercress resulted in a mean of 0.9 metacercariae per bed of watercress over a 9 year period (Dreyfuss, Vignoles, & Rondelaud, 2005). Similarly low numbers of metacercariae were recorded on watercress beds in a more recent study, performed over 14 years. On the year with the highest count, 1999, a mean of 4.6 metacercariae was observed over 32 beds of watercress (Dreyfuss, Vignoles, & Rondelaud, 2005). This study used simple microscopy to identify metacercariae on the watercress. Considering the volume of herbage that would have been inspected and the knowledge that metacercariae appear almost translucent on any herbage making it very difficult to identify them, it is possible that some metacercariae may have been missed. There is not any published evidence detailing the number of *F. hepatica* metacercariae or potential infection load on pasture. So it is unclear if the numbers observed in this study are a true representation of what would occur in the environment.

Instead of painstaking identification of snails *in situ* and/or metacercariae from pasture, the only outdoor work required for DNA detection of either *F. hepatica* and or *G. truncatula* would be the collection of grass samples. Many samples of grass could be collected from a field, covering all areas regardless of the perceived suitability of snail habitat. This allows for greater coverage of a field with the capacity for large-scale processing. From the resulting data it would be possible to pin point exact areas of fields where fluke has been identified. Farmers could then fence off areas of high risk or not use a high risk field for grazing a way of avoiding fasciolosis in their herd.

Another advantage of a DNA based test is the ability to identify *F. hepatica* specifically, accurately differentiating from other fluke species. Many different species of parasitic Platyhelminthes exist in the same or similar environments as *F. hepatica* whilst also utilizing the same intermediate and definitive hosts. *C. daubneyi* is a prime example; this species has been shown to utilize *G. truncatula* as an intermediate host (Jones et al., 2015). Co-infections of snails with both C. daubneyi and *F. hepatica* have been observed in France (Rondelaud, Vignoles, & Dreyfuss, 2004) and in Wales (Jones et al., 2015).

Determining species identity of any metacercariae isolated from pasture is important for the correct diagnosis of disease risk. Incorrect identification could lead to unnecessary and potentially damaging implementation of disease avoidance techniques and targeted treatment. Farmers cannot afford to waste time, money and effort implementing avoidance strategies such as the moving of livestock to cleaner pastures or treating whole flocks if there is no risk of fasciolosis. The problem of anthelmintic resistance could also be exacerbated if farmers misuse flukicides after following the suggested pre-cautionary actions of an inaccurate fasciolosis forecast.

DNA analysis also allows for the testing of multiple different types of samples. Water and grass silage are other potential sources of infections for livestock. *F. hepatica* environmental DNA or eDNA has been identified from water collected from a field harbouring infected *G. truncatula* (Jones et al, 2018). This particular project used water samples to detect *F. hepatica* in the environment. Metacercariae are known to encyst on water surfaces and form aggregates (Abrous et al., 2001). It is possible that an animal could become infected by drinking contaminated water but the level of threat of infection from water sources is unknown. It is more likely that livestock will be infected by grazing contaminated pasture. Metacercariae are most likely to encyst on the underside of green leaves or stems over brown leave or the water surface (Hodasi, 1972). Not only are the metacercariae more likely to be consumed but this preference is beneficial to the survival of the metacercariae as on the underside of leaves metacercaria are less likely to desiccate or be damaged by environmental factors.

Metacercariae are able to survive the fermentation conditions of grass silage for 4 weeks (Chapter 6). DNA testing was not performed on any silage samples but it is believed that LAMP would have no difficulty identifying the COX1 target in silage samples due to the high similarity with fresh grass samples. The possibility of an animal developing fasciolosis from consuming contaminated silage is recognised but it is unknown how many cases have resulted from such scenarios.

Farmers can avoid fasciolosis in the stock by avoiding high risk pasture. Other methods of disease avoidance include the reduction of *G. truncatula* numbers through drainage of habitat. Molluscicides were previously implemented but are now banned because of the potential harm to the wider environment that they pose (Torgerson & Claxton, 1998). Reducing water from the surface and sub-surface of a field using drainage offers a less dangerous method for the removal of *G. truncatula* habitat. Where the removal of snail habitat is not possible, livestock can be prevented from grazing such areas by use of fencing and or moving them to low risk pasture.

In some west, upland areas of Scotland most of the grazing pasture could be deemed suitable habitat for *G. truncatula* and therefore identified as high risk for fasciolosis. Farmers in these areas may not have the option to graze their animals on low risk pasture and drainage may not be an economically viable option. In this situation farmers are advised to ‘protect’ pasture by reducing the egg output onto fields, thus reducing the risk of snails becoming infected.

Current understanding of liver fluke epidemiology is less than desirable and the tools used to avoid disease are not always suitable nor economically viable, especially if all grazing land is deemed high risk (Sargison, 2014). DNA testing could potentially help farms in this situation. Greater accuracy in determining where *F. hepatica* is in a field is achievable with DNA testing and this is beneficial for farmers as they can fence off or drain these areas without discounting the whole field as unsuitable for grazing. This is particularly beneficial for those farms where the majority or all of the grazing land might be deemed high risk from visual inspection and snail surveys.

LAMP has the potential to be transformed into an on farm-based test. For practicality and ease of use on farm, the current method of detecting the amplified target would need to be altered. Instead of using an intercalating fluorochrome dye (MAST), a fluorescence dye could be used to measure the amplification of the target from samples. The appearance of fluorescence within a sample tube can be detected by eye. This is beneficial for the development of a farm-based test as this does not require the use of RT-PCR thermo cyclers which are expensive and not suitable for easy determination of sample status by potential none lab based end point users. This method of LAMP has already been successfully demonstrated for the detection of *F. hepatica* ITS-2 target from faecal samples 3 weeks post infection (Gordon-Gibbs, 2014).

There are also limitations for the use PCR and LAMP in determining fasciolosis risk on pasture. As the origin of the amplified DNA cannot be known, any results should be considered in relation to any metacercarial recovery data, the fasciolosis status of stock on the farm, the presence and status of *G. truncatul*a on the field. The epidemiological strength of any theoretical results gathered from pasture samples would be poor, especially considering the amount of processing these methods require. Without physical confirmation of metacercariae on the pasture by their physical recovery, any DNA positive results are effectively meaningless from a *F. hepatica* risk perspective. *F. hepatica* COX 1 amplification can offer a quick, but crude pin point determination of *F. hepatica* present in a field or on a farm but does not inform the user as to the number or viable metacercariae present. However, positive and or negative confirmation of *F. hepatica* presence on fields/parts of fields does benefit some farms where confirmation of any low risk grazing would be helpful.

In Chapter 5, both PCR and LAMP were demonstrated to be capable of detecting very small amounts of metacercarial DNA from spiked grass. Jones et al., (2018) has already demonstrated eDNA methods for detection of *F. hepatica* DNA from water and pasture. This study also acknowledges that although this proof-of-concept for DNA based detection of *F. hepatica* from the environment is achievable, the very nature of working with environmental samples is challenging. Effective DNA extraction is influenced by the eDNA filters which are subject to clogging. This influences the sensitivity of the assays as the volume of sample that can be processed is limited by the amount of sample that can pass through the filter. Also highlighted was the knowledge that a positive result does not always equate to physical presence nor does a negative result equate to absence.

The amount of grass needed to determine the presence of *F. hepatica* on a field is considerable. Indeed, the MAFF (1986) method of metacercarial recovery from pasture requires 100g of grass per individual sample. Recognising that many hundreds of grams of grass would be required to survey a complete field amplifies the workload considerably.

Practical application of pasture DNA analysis to detect *F. hepatica* on this volume of grass is not realistic or appropriate for determining fasciolosis risk. Alternative methods of sample processing and DNA extraction that could handle larger samples sizes efficiently and at low cost would be the ideal solution. However, the more valuable solution would be focus on further development of efficient method for the recovery of wild metacercariae from pasture samples. The species of metacercariae recovered in the method of metacercarial recovery explained in section 6.2 could be confirmed by the use of the LAMP assay described in Chapter 5.

6.5 Conclusion

This project has highlighted that there is not a singular, all-encompassing method or answer for the determination of liver fluke risk on farms. For an accurate assessment of fasciolosis risk on farms all information must be considered, including: knowledge of the history of fasciolosis on farm, the current status of livestock, treatment plans, snail surveys and weather readings. All of this information, including the viability and quantity of metacercariae from pasture can be combined from farms across the country to build up a more comprehensive understanding of liver fluke epidemiology and more accurate predictions of disease out breaks on individual farms in the U.K.

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Appendices

Appendix 1

Modified DNeasy Blood & Tissue Kit protocol

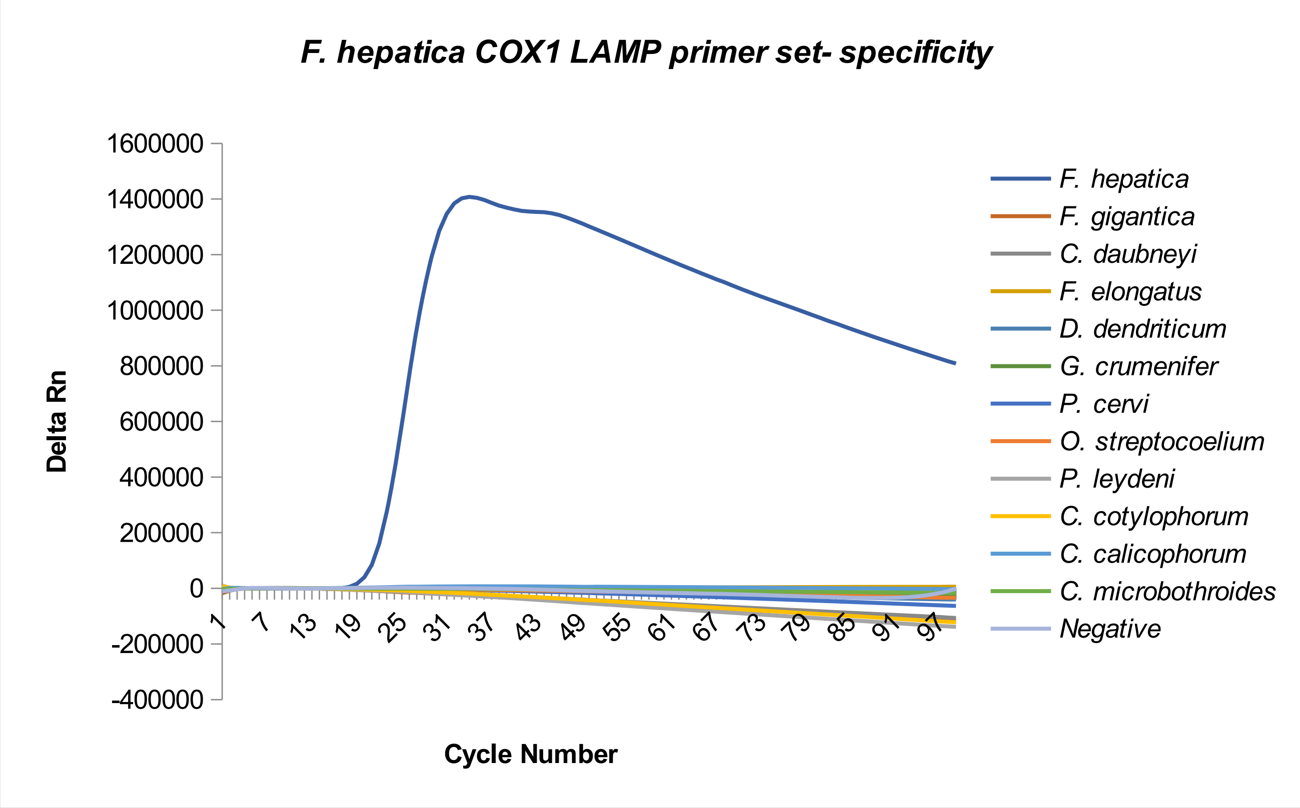
Place metacercariae or 0.05g of grass in a in a 1.5 ml Eppendorf tube. Add 180µl Buffer ATL and 20µl proteinase K. Vortex for 15 s and incubate in a water bath 56˚C from 4 hours - overnight to completely lyse tissue.
Dry sample tubes and vortex for 15 seconds and add 200µl Buffer AL, vortex, add 200µl 100% ethanol and vortex.

Pipette mixture into a DNeasy Mini spin column in a 2 ml collection tube. Centrifuge at 6,000 g for 1 minute. Discard flow through and collection tube.
Place the DNeasy Mini spin column in a new 2 ml collection tube.Add 500µl Buffer AW1, centrifuge for 1 minute at 6,000 g. Discard flow through and spin column.

Place the DNeasy Mini spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2, centrifuge for 4.5 minutes at 15,000 g. Discard flow through and spin column.

Place the DNeasy Mini spin column in a clean 1.5 ml centrifuge tube.Pipette 100µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute.Centrifuge at 6,000 g for 1 minute to elute the DNA.
Store DNA at -20˚C.

Appendix 2



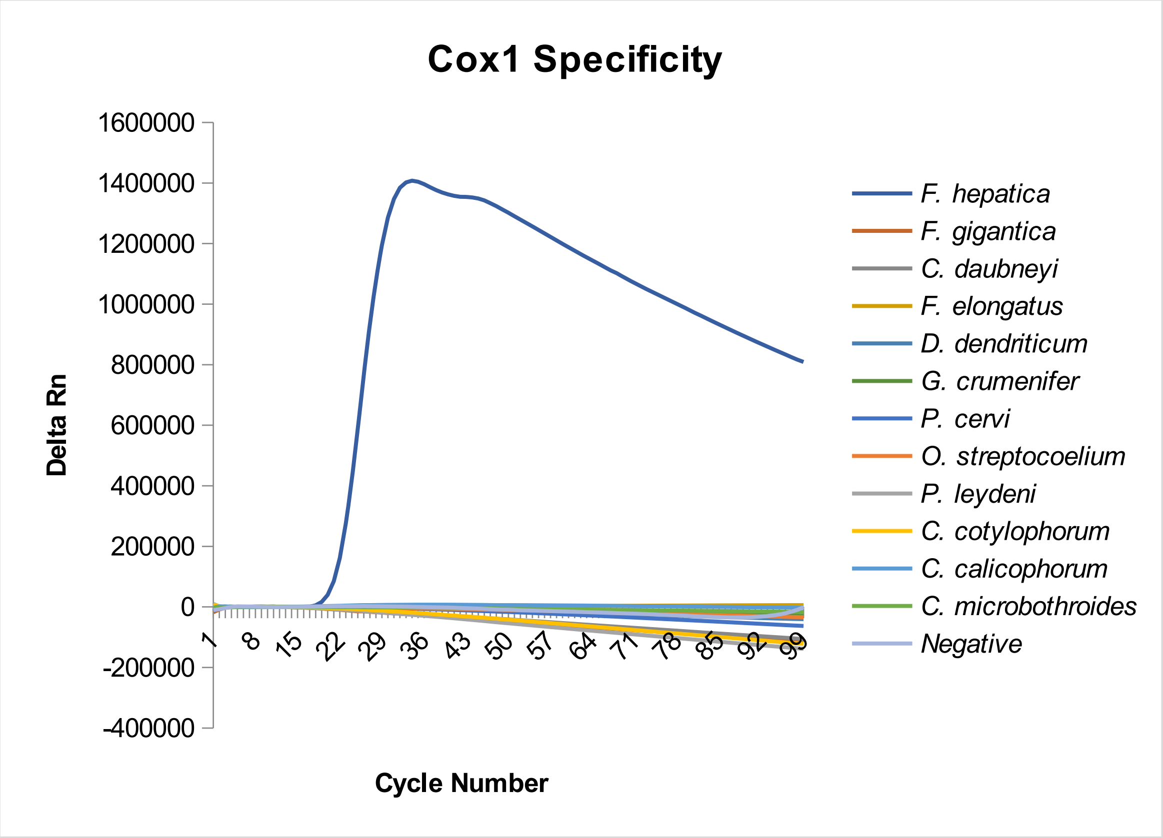
F. hepatica COX1 LAMP primer set- specificity
Delta Rn
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
-400000
1
7
13
19
25
31
37
43
49
55
61
67
73
79
85
91
97
Cycle Number
F. hepatica
F. gigantica
C. daubneyi
F. elongatus
D. dendriticum
G. crumenifer
P. cervi
O. streptocoelium
P. leydeni
C. cotylophorum
C. calicophorum
C. microbothroides
Negative

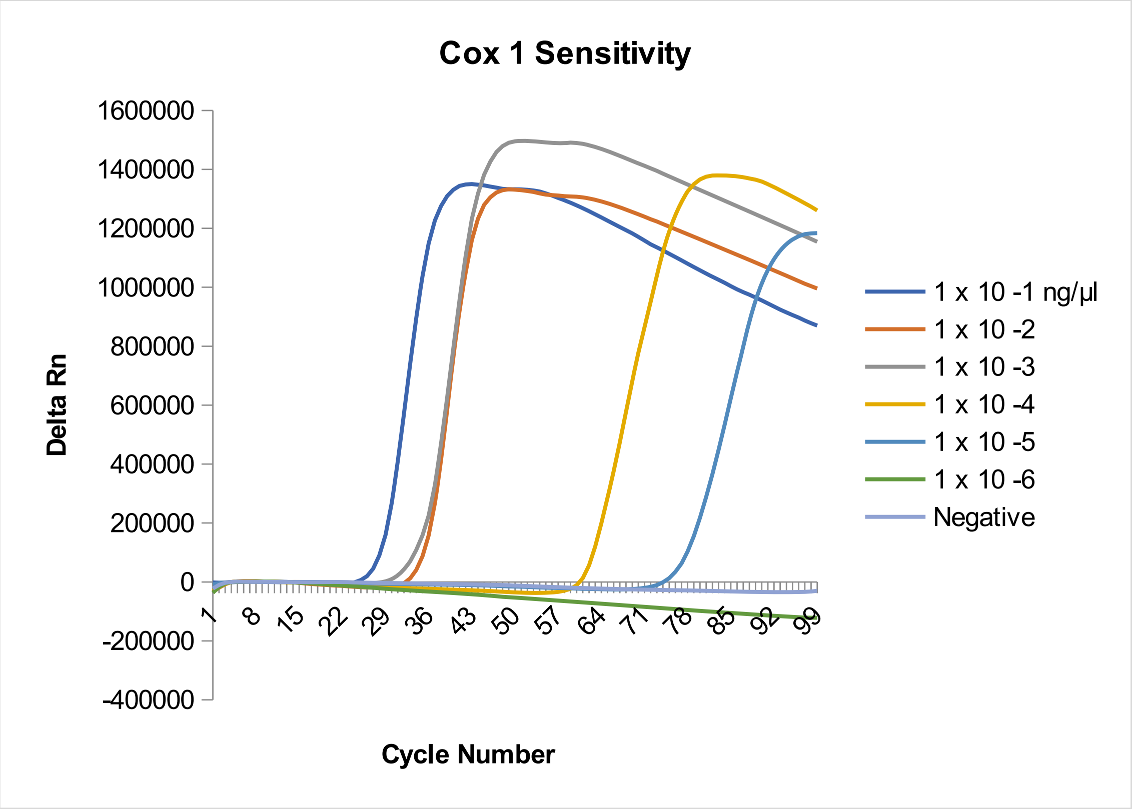
Appendix 3

Table appendix 3 – CT values and corresponding time to detection

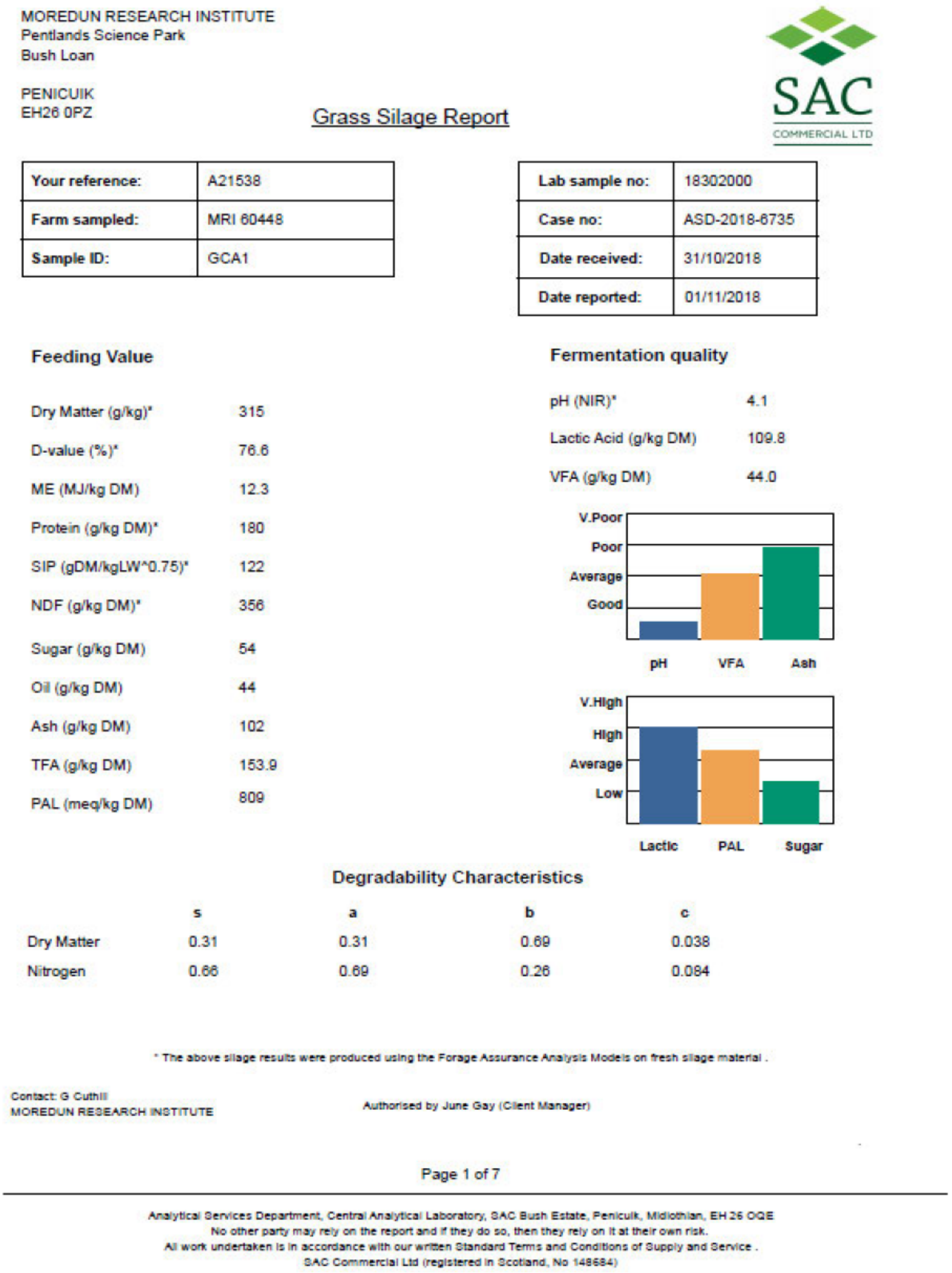
DNA samples	CT values	Time to detection (minutes)
1	37	20
	29	16
	30	17
10	22	12
	23	13
	27	15
20	27	15
	24	13
	24	13
30	99	54
	28	15
	29	16
10 ng/µl	19	11
	21	12
	19	11
NTC	99	54
	99	54
	99	54

Appendix 4


Cox1 Specificity
Delta Rn
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
-400000
1
8
15
22
29
36
43
50
57
64
71
78
85
92
99
Cycle Number
F. hepatica
F. gigantica
C. daubneyi
F. elongatus
D. dendriticum
G. crumenifer
P. cervi
O. streptocoelium
P. leydeni
C. cotylophorum
C. calicophorum
C. microbothroides
Negative


Cox 1 Sensitivity
Delta Rn
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
-400000
1
8
15
22
29
36
43
50
57
64
71
78
85
92
99
Cycle Number
1 x 10 -1 ng/µl
1 x 10 -2
1 x 10 -3
1 x 10 -4
1 x 10 -5
1 x 10 -6
Negative

Appendix 5



MOREDUN RESEARCH INSTITUTE
Pentlands Science Park
Bush Loan

PENICUIK
EH26 0PZ

SAC
COMMERCIAL LTD

Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCA1

Lab sample no:	18302000
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

Feeding Value

Dry Matter (g/kg)*	315
D-value (%)*	76.6
ME (MJ/kg DM)	12.3
Protein (g/kg DM)*	180
SIP (gDM/kgLW^0.75)*	122
NDF (g/kg DM)*	356
Sugar (g/kg DM)	54
Oil (g/kg DM)	44
Ash (g/kg DM)	102
TFA (g/kg DM)	153.9
PAL (meq/kg DM)	809

Fermentation quality

pH (NIR)*	4.1
Lactic Acid (g/kg DM)	109.8
VFA (g/kg DM)	44.0

Degradability Characteristics

	s	a	b	c
Dry Matter	0.31	0.31	0.69	0.038
Nitrogen	0.66	0.69	0.26	0.084

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

Contact: G Cuthill
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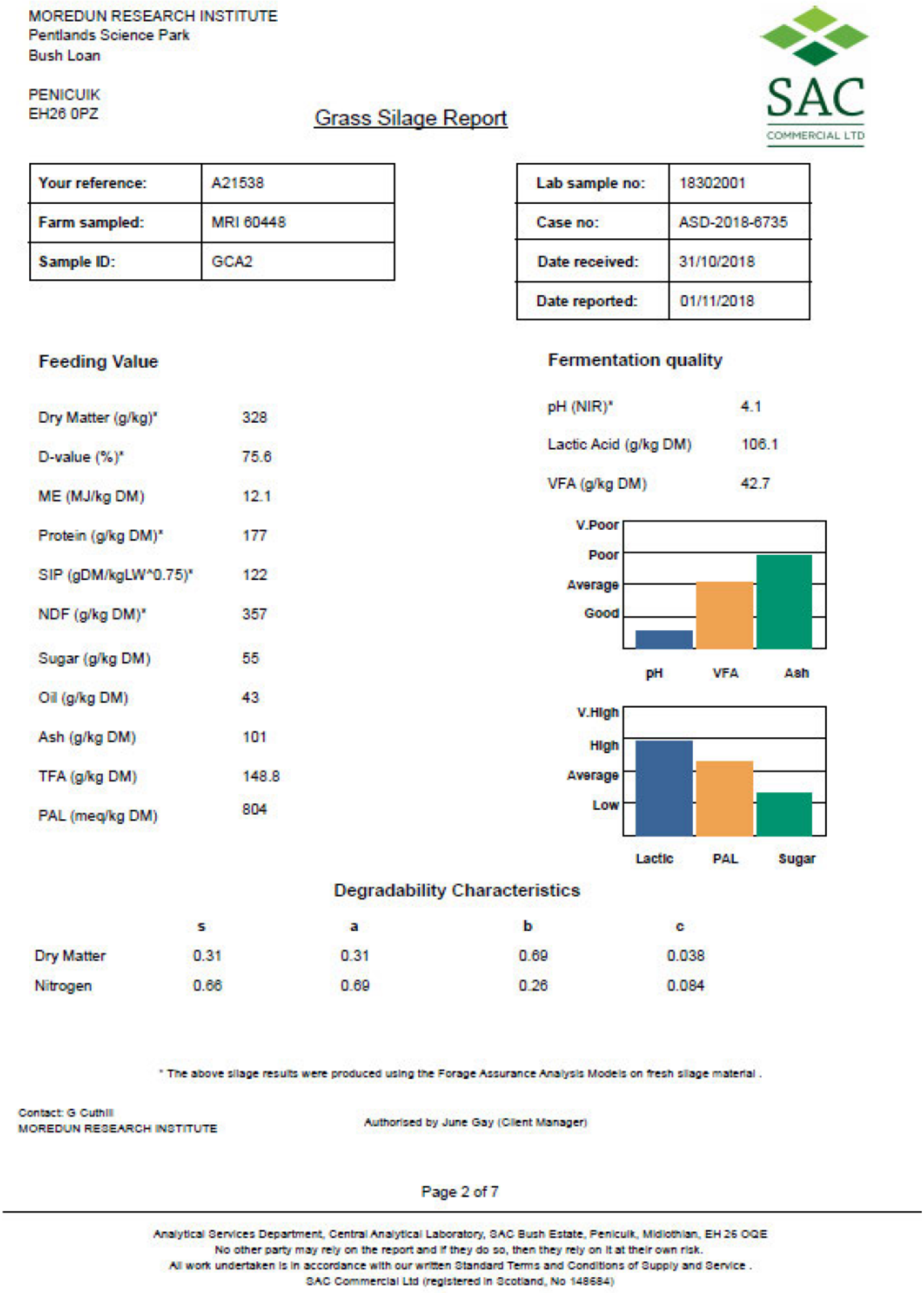

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Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCA2

Lab sample no:	18302001
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

Feeding Value

Dry Matter (g/kg)*	328
D-value (%)*	75.6
ME (MJ/kg DM)	12.1
Protein (g/kg DM)*	177
SIP (gDM/kgLW^0.75)*	122
NDF (g/kg DM)*	357
Sugar (g/kg DM)	55
Oil (g/kg DM)	43
Ash (g/kg DM)	101
TFA (g/kg DM)	148.8
PAL (meq/kg DM)	804

Fermentation quality

pH (NIR)*	4.1
Lactic Acid (g/kg DM)	106.1
VFA (g/kg DM)	42.7

Degradability Characteristics

	s	a	b	c
Dry Matter	0.31	0.31	0.69	0.038
Nitrogen	0.66	0.69	0.26	0.084

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

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Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCB1

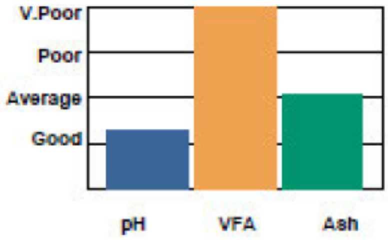
Lab sample no:	18302002
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

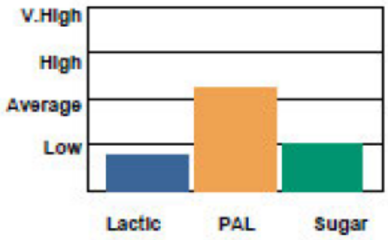
Feeding Value

Dry Matter (g/kg)*	281
D-value (%)*	63.9
ME (MJ/kg DM)	10.2
Protein (g/kg DM)*	131
SIP (gDM/kgLW^0.75)*	103
NDF (g/kg DM)*	537
Sugar (g/kg DM)	46
Oil (g/kg DM)	36
Ash (g/kg DM)	78
TFA (g/kg DM)	97.4
PAL (meq/kg DM)	791

Fermentation quality

pH (NIR)*	4.7
Lactic Acid (g/kg DM)	21.7
VFA (g/kg DM)	75.7





Degradability Characteristics

	s	a	b	c
Dry Matter	0.27	0.34	0.57	0.036
Nitrogen	0.58	0.68	0.23	0.079

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

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Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCB2

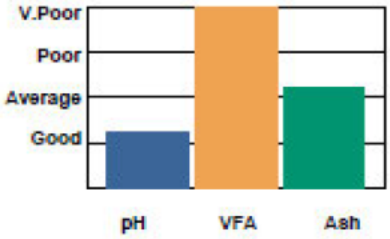
Lab sample no:	18302003
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

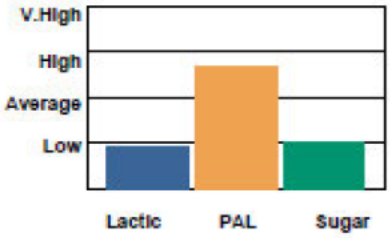
Feeding Value

Dry Matter (g/kg)*	261
D-value (%)*	65.5
ME (MJ/kg DM)	10.5
Protein (g/kg DM)*	134
SIP (gDM/kgLW^0.75)*	103
NDF (g/kg DM)*	513
Sugar (g/kg DM)	46
Oil (g/kg DM)	38
Ash (g/kg DM)	80
TFA (g/kg DM)	106.7
PAL (meq/kg DM)	847

Fermentation quality

pH (NIR)*	4.6
Lactic Acid (g/kg DM)	26.1
VFA (g/kg DM)	80.7





Degradability Characteristics

	s	a	b	c
Dry Matter	0.27	0.34	0.57	0.036
Nitrogen	0.58	0.68	0.23	0.079

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

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Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCC1

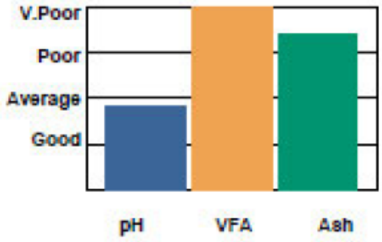
Lab sample no:	18302004
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

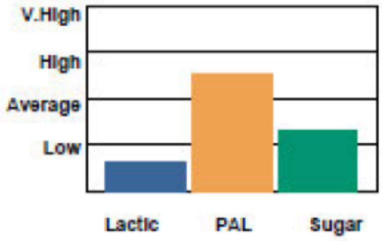
Feeding Value

Dry Matter (g/kg)*	255
D-value (%)*	58.7
ME (MJ/kg DM)	9.4
Protein (g/kg DM)*	189
SIP (gDM/kgLW^0.75)*	98
NDF (g/kg DM)*	439
Sugar (g/kg DM)	55
Oil (g/kg DM)	35
Ash (g/kg DM)	118
TFA (g/kg DM)	77.3
PAL (meq/kg DM)	825

Fermentation quality

pH (NIR)*	5.2
Lactic Acid (g/kg DM)	15.7
VFA (g/kg DM)	61.6





Degradability Characteristics

	s	a	b	c
Dry Matter	0.22	0.32	0.51	0.036
Nitrogen	0.51	0.65	0.24	0.066

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

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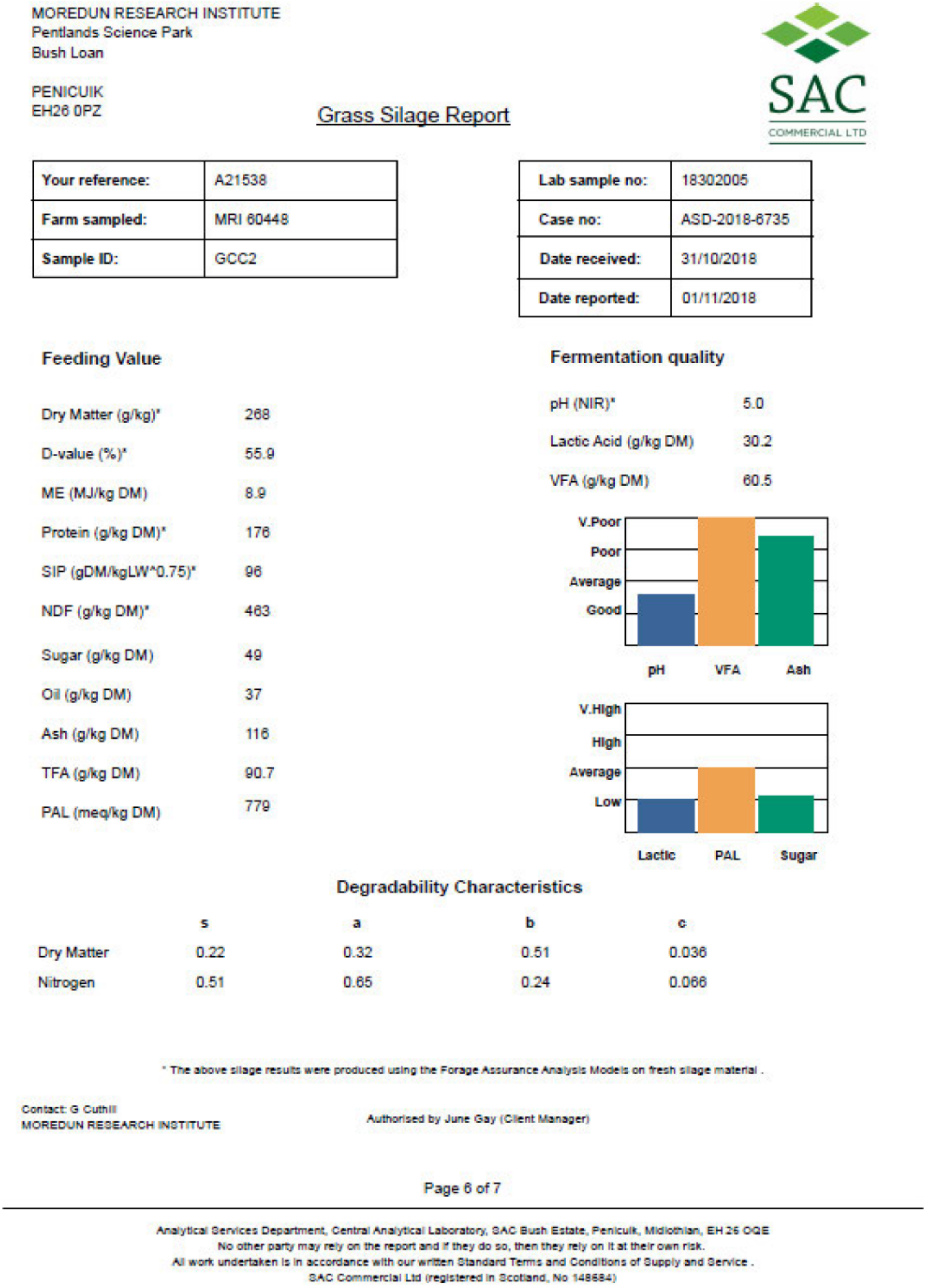

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Grass Silage Report

Your reference:	A21538
Farm sampled:	MRI 60448
Sample ID:	GCC2

Lab sample no:	18302005
Case no:	ASD-2018-6735
Date received:	31/10/2018
Date reported:	01/11/2018

Feeding Value

Dry Matter (g/kg)*	268
D-value (%)*	55.9
ME (MJ/kg DM)	8.9
Protein (g/kg DM)*	176
SIP (gDM/kgLW^0.75)*	96
NDF (g/kg DM)*	463
Sugar (g/kg DM)	49
Oil (g/kg DM)	37
Ash (g/kg DM)	116
TFA (g/kg DM)	90.7
PAL (meq/kg DM)	779

Fermentation quality

pH (NIR)*	5.0
Lactic Acid (g/kg DM)	30.2
VFA (g/kg DM)	60.5

Degradability Characteristics

	s	a	b	c
Dry Matter	0.22	0.32	0.51	0.036
Nitrogen	0.51	0.65	0.24	0.066

* The above silage results were produced using the Forage Assurance Analysis Models on fresh silage material .

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Explanation of Silage Terms

Dry Matter	the non-water part of the sample. Shows a very wide range depending on wilting period and the weather. Target for clamps is about 260 and 350 for big bales provided wilting can be achieved quickly.
Metabolisable Energy	measures the useful energy in the silage allowing for losses in faeces, urine and methane. Calculated from the D value using standard UK equations. Range is 8.0-12.5. Depends mostly on crop maturity at cutting.
SIP	shows the intake potential of the silage as a sole feed. Depends on DM content, D value and fermentation characteristics. Range 60-120. Average silage has a SIP of approx. 90.
Protein	calculated from total nitrogen, includes non-protein as well as true protein. Values vary between 100 - 200. Depends on type of crop, maturity, fertiliser, weather.
NDF	neutral detergent fibre - the amount of fibre (cell walls) in the silage. Used in ration calculations to help avoid digestive upsets (due to low rumen pH) by getting the right balance between quickly and slowly digested feeds. Range 300 - 700.
D Value	the digestibility of the silage (as the % digestible organic matter in the dry matter). Depends mostly on crop maturity but reduced by high ash content. Range 45 - 78.
sDm, aDM, bDM, cDM	used to describe the rate and extent of DM degradation in the rumen. High D value silages have greater b and c terms. Used in FeedByte to calculate how much energy the rumen bacteria can get from the silage. The greater the energy supply the greater the need for degradable protein and the greater the potential supply of micobial protein
sN, aN, bN, cN	used to describe the rate and extent of nitrogen degradation in the rumen. Used in FeedByte to calculate how much protein breaks down in the rumen and is potentially available to the rumen bacteria and how much by-passes the rumen for digestion in the intestines.
pH	a measure of acid balance and a good indicator of whether a stable preservation has been achieved. The pH histogram allows for the need for lower pH values for wetter silages. Range 3.4 - 7.0.
VFA	measures the concentration of undesirable volatile fatty acids. The lower the value the more efficient the fermentation has been. High values (>40) are usually associated with high nutrient losses, protein breakdown and poor intakes. Range 0-100. Good silage <20.
PAL	the potential acid load of the silage. Range 600 -1200. This depends on the acidity of the silage and the amount of acid produced in the rumen. Acidic, high D silages have high values. **(Values over 900 could increase the risk of digestive upsets - seek nutritional advice)**
Lactic	the acid which is normally responsible for preservation. Badly fermented silages often have low levels but this depends on dry matter content and additive use. Very high levels (>120) found in over-fermented acid silages and can reduce intake. Range 0-180.
Sugar	the sugar remaining after fermentation. High levels (>100) are generally desirable resulting from a restricted fermentation (caused by wilting or use of some additives) of a high-sugar crop. Low levels may indicate an extensive fermentation and should not be considered bad unless associated with poor ratings for pH, NH3, and VFA. Range 0-250.
Ash	the mineral content of the silage - high levels (>80) indicate soil contamination which increases the risk of a bad fermentation. High ash levels lower the ME and D values.Range 40-200.

Research Dissemination

Proposed publications

- “Development of DNA assays for the identification of *F. hepatica* metacercariae from pasture samples”

- “Survival of *F. hepatica* metacercariae within grass silage and at pH associated with grass silage fermentation”

Presentations

Ninth International Sheep Veterinary Congress 22nd-26th May 2017;

- 8 minute presentation during session ‘*Global challenges due to small ruminant trematode parasites’* - “Identification of viable liver fluke and rumen fluke metacercarial challenge to grazing livestock”
- Poster presentation – *“In vitro* evaluation of the effect of pH on *F. hepatica* metacercarial viability”

World Association for the Advancement of Veterinary Parasitology (WAAVP) 2th-8th September 2017- Rapid, 2 minute, presentations;

- “*In vitro* evaluation of the impact of pH on *Fasciola hepatica* metacercarial viability”
- “Diagnosis of fluke infective stages in the environment”

AHDB

- 7/12/15; 5 minute presentation – “Identification of viable liver fluke and rumen fluke metacercarial challenge to grazing livestock”
- 15/11/16; Poster – “Diagnosis of fluke infective stages in the environment”
- 9/11/17; Poster – “Diagnosis of fluke infective stages in the environment”

- 2018; 15 minute final year presentation – “Diagnosis of fluke infective stages in the environment”

Co-authorship

Mitchell, G., Cuthill, G., Haine, A., Zadoks, R., Chaudhry, U., Skuce, P and Sargison, N. (2017). Evaluation of molecular methods for the field study of the natural history and changing epidemiology of *Dicrocoelium dendriticum. Veterinary Parasitology.* 235. 100-105. https://doi.org/10.1016/j.vetpar.2017.01.010.