Population genetic structure of *Fasciola hepatica* in Great Britain

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by

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The liver fluke, *Fasciola hepatica*, is a trematode parasite that causes disease of economic and welfare importance to the UK livestock sector. Prevalence of *F. hepatica* infection in sheep and cattle in the UK is increasing; in part due to changes in climate and farming practice, but compounded by the emergence of resistance to the drug triclabendazole. Adult *F. hepatica* within the definitive host are hermaphrodite, capable of both self- and cross-fertilisation. There are reports of parasite populations maintained by parthenogenesis. Diploid (2n = 2x = 20) and triploid (2n = 3x = 30) *F. hepatica* have been reported in the UK, but there is little information to indicate how frequently triploidy occurs.

The *F. hepatica* life cycle is complex requiring a snail intermediate host, *Galba truncatula*. Clonal expansion of the parasite occurs within the snail followed by release of cercariae which subsequently encyst on pasture. Little is known about the population genetic structure of *F. hepatica* in the UK, or how the clonal expansion in the snail, the capacity for self-fertilisation, and the potential for aggregation of metacercariae on pasture, influence genetic diversity of *F. hepatica* populations within the definitive host. Knowledge of the genetic structure of *F. hepatica* in Great Britain is critical to understanding the level of gene flow within *F. hepatica* populations and how this impacts on the spread of drug resistance genes. The aim of this thesis was to determine the ploidy and population genetic structure of *F. hepatica* infecting sheep and cattle in Great Britain, and define how passage of the parasite through the snail intermediate host may influence genetic diversity.

To determine ploidy a total of 715 adult parasites were collected from naturally infected sheep and cattle, exceeding the statistically representative sample size of 384 individuals (expected proportion of triploids 50 %; 95 % confidence level; 5 % confidence limits). The ploidy and presence of sperm was determined by aceto-orcein squash. One hundred percent were confirmed as diploid and all contained sperm. A multiplex PCR and capillary electrophoresis approach was validated for a panel of fifteen polymorphic microsatellites to assess the population genetic structure of *F. hepatica*. Eight of these microsatellites met the criteria for population genetic analyses, and were used to produce a multilocus genotype (MLG) for 950 adult parasites from the livers of 44 naturally infected sheep, and 629 *F.*

hepatica adults from 31 naturally infected cattle livers. The average heterozygosity across all parasites and loci was determined as 0.752 (SD = 0.130), indicating high levels of genetic variation in *F. hepatica* populations. Of the 1579 samples, 1424 distinct MLGs were observed, which supports high genotypic diversity in the population as a whole. Forty six hosts harboured multiple, genotypically identical parasites, which were shown to have arisen from clonal lineages rather than distinct reproductive events. The F_{IS} value indicates a self-fertilisation rate no higher than 2 %, and the F_{ST} value (0.0202) indicates low population structure and high gene flow in the British *F. hepatica* population.

To assess the influence of the snail intermediate host on genetic diversity, MLG profiles were used to address: (i) whether a snail can be infected with, and shed cercariae of, more than one genotype of *F. hepatica* and (ii) the population structure of *F. hepatica* within field populations of snails in Great Britain. For experiment (i) 49 *G. truncatula*, from a colony maintained in the laboratory, were exposed to miracidia of known, but distinct, genotype. Two snails, showed evidence of infection with more than one genotype of *F. hepatica*, but did not shed cercariae. In experiment (ii) 155 snails, including 52 *G. truncatula*, were collected from three farms in Scotland and North Wales. None of the snails showed evidence of infection with *F. hepatica*.

The results here show that *F. hepatica* isolated from British cattle and sheep are diploid. In addition, genetic analysis suggests cross-fertilisation predominates over self-fertilisation and parthenogenesis. The British *F. hepatica* population shows high genetic diversity within populations, but little genetic differentiation between populations, and is therefore panmictic. Our analyses support high gene flow and identified a proportion of parasites within the definitive host that are genetically identical. These observations suggest that drug resistance genes could spread rapidly in *F. hepatica* populations.

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I have made lifelong friends during my three and a bit years studying for my PhD. There have been serious discussions and advice, jokes, baby showers, marriages, conferences, and an awful lot of cake. In alphabetical order (as per JK) thanks to: Alison, Cara, Christina, Corrado, Dan, Elsa, Isabel, Jess, John, Juriah, Laura, Leigh, Maria, Patrick, Ross, Sara, Stefan, Tess, and Tonk.

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For Pom-Pom, who would have been proud of his Nicola Jane

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^{o}C	Degrees Celsius
β	Slope of the fitted log-log regression equation
μgml^{-1}	Microgram per millilitre
µl	Microlitre
µm	Micrometre
µM	Micromolar
X^2	Chi-Square test statistic
APHA	Animal and Plant Health Agency
BBSRC	Biotechnology and Biological Sciences Research Council
bp	Base pairs
CI	Confidence interval
cm	Centimetre
*COX*1	Cytochrome c oxidase I
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
D_A	Measure of genetic difference
dpi	Days post infection
FECRT	Faecal egg count reduction test
g	G-force
GB	Great Britain
h	Hour(s)
H_{nb}	Unbiased heterozygosity
H_{obs}	Observed heterozygosity
HAIRS	Human Animal Infections and Risk Surveillance
ITS-1	Internal transcribed spacer 1
ITS-2	Internal transcribed spacer 2
K	Number
kg	Kilogram
m^2	Metres squared
ml	Millilitre
MLG	Multilocus genotype
mm	Millimetre

mm^3	Millimetre cubed
mM	Millimolar
MT_a	Multiplex annealing temperature
mtDNA	Mitochondrial DNA
n / No.	Number
N_m	Number of migrants between populations
*NAD*1	NAD(P)H dehydrogenase 1
*NAD*3	NAD(P)H dehydrogenase 3
NADIS	National Animal Disease Information Service
ND	Not determined
ng	Nanogram
$ng\mu l^{-1}$	Nanogram per microlitre
$ngml^{-1}$	Nanogram per millilitre
p	*P*-value
P_{sex}	probability that a MLG is derived from a distinct reproductive event rather than being from the same clonal lineage
PCA	Principle component analysis
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
R	Genotypic richness
r^2	Extent of linkage disequilibrium / Regression co-efficient
rDNA	Ribosomal DNA
RADAR	Rapid Analysis and Detection of Animal Related Risks
RAPD	Random amplified polymorphic DNA-PCR
RFLP-PCR	Restriction fragment length polymorphism PCR
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RT	Room temperature
s	Rate of self-fertilisation
SD	Standard deviation
SNP	Single nucleotide polymorphism
sp. / spp.	Species
SRAP	Sequence-related amplified polymorphism

ST_a	Singleplex annealing temperature
$U\mu l^{-1}$	Units per microlitre
UK	United Kingdom
USA	United States of America
V	Volt
VIDA	Veterinary Investigation Diagnosis Analysis
w/v	Weight / volume
WHO	World Health Organisation
x	Basic chromosome number

CHAPTER 1

Introduction

1.1 The importance of Fasciola hepatica

Fasciolosis is a disease of livestock and humans caused by trematode parasites of the genus *Fasciola*. The two species that most commonly cause disease are *Fasciola hepatica* and *Fasciola gigantica*. Since the adult parasites reside in the bile ducts of the definitive host these parasites are commonly referred to as liver flukes. Although *F. hepatica* has a worldwide distribute, it predominates in temperate areas, and *F. gigantica* is primarily found in tropical areas. Therefore the two species overlap in areas of Asia and Africa. In the UK, the only species that occurs is *F. hepatica* and here the parasite predominately infects sheep and cattle. It is estimated that 250 million sheep and 350 million cattle worldwide are at risk of disease caused by *F. hepatica* (Hillyer and Apt, 1997).

Three forms of disease are recognised in livestock: acute, sub-acute and chronic; and production losses can be seen even if the disease is subclinical. These losses, as well as the costs associated with treatment and control, mean that fasciolosis is an economically important disease. Economic losses have been estimated to be up to €92 million per annum in Switzerland (Schweizer *et al.*, 2005) and up to £40.4 million per annum in Great Britain (Bennett and Ijpelaar, 2005). It is known that *F. hepatica* also has the ability to modulate the immune system and alter the immune response of the definitive host to other pathogens. This may make the host more susceptible to other pathogens, or alter the results of diagnostic tests (Claridge *et al.*, 2012). Recent reports of the seroprevalence of *F. hepatica* in dairy cattle herds in Britain, determined from bulk milk tank samples, found similar prevalence levels, 76 % and 79.7 %, with little change over time (McCann *et al.*, 2010a; Howell *et al.*, 2015). In the Republic of Ireland prevalence of *F. hepatica* in sheep, based on faecal egg counts, has been reported to be 61.6 % (Rinaldi *et al.*, 2015). Over the last 15 to 20 years diagnosis of *F. hepatica* in livestock in the UK has increased (VIDA, 2015). This increased occurrence is likely to be multifactorial, but it has been suggested that this may be due to changing climate, changing farming practices, including in animal movements; and the emergence of resistance to the drug triclabendazole, the treatment of choice for this parasite. (van Dijk *et al.*, 2010; Fairweather, 2011a; Fox *et al.*, 2011; Caminade *et al.*, 2015).

Evidence of *F. hepatica* infection has been found in prehistoric human populations (Aspöck *et al.*, 1999), and human fasciolosis is still a problem in the

modern developing world: the disease is classified by the World Health Organisation as a neglected tropical disease (WHO, 2015). In recent years there have only been a handful of cases in humans in Great Britain (HAIRS, 2010), yet worldwide an estimated 2.4 million people are infected with *F. hepatica*, with up to 180 million people at risk of developing infection (WHO, 2007). A number of epidemiological classifications of human fasciolosis exist: imported cases, autochthonous or isolated cases; hypo-, meso- and hyper-endemic cases; and epidemic cases in either human or animal endemic areas. Current areas where human fasciolosis is considered to be endemic are Bolivia, Peru, Chile, Ecuador, Cuba, Egypt, western Europe (Portugal, France and Spain), and the Caspian (Iran and neighbouring countries; Mas-Coma, 2005; WHO, 2007).

1.2 The life cycle of Fasciola hepatica

The life cycle of *F. hepatica*, elucidated by Thomas, (1883) is a complicated two-part life cycle, with development taking place on pasture and within a snail intermediate host (Figure 1.1).

1.2.1 Development and survival of Fasciola hepatica *eggs*

Adult *F. hepatica* live in the bile ducts of the definitive host; they are hermaphrodite so can self- and cross-fertilise to produce eggs. These undifferentiated eggs are passed onto pasture in the faeces. Eggs of *F. hepatica* do not develop well in faeces and must be liberated from the faecal pat. They are prone to desiccation, but soil moisture helps prevent this (Ollerenshaw, 1959). The egg develops on pasture to the next life cycle stage: a miracidium. The rate of this development is related to temperature: development is inhibited at low (below 9.5^{o}C) and high (above 30^{o}C) temperatures, and is fastest between 10 and 25^{o}C (Rowcliffe and Ollerenshaw, 1960; Boray, 1969). At a constant temperature of 26^{o}C eggs can fully develop within 12 days, but under natural conditions, where temperatures fluctuate, development can take anywhere from 21 to 100 days (Boray, 1969).

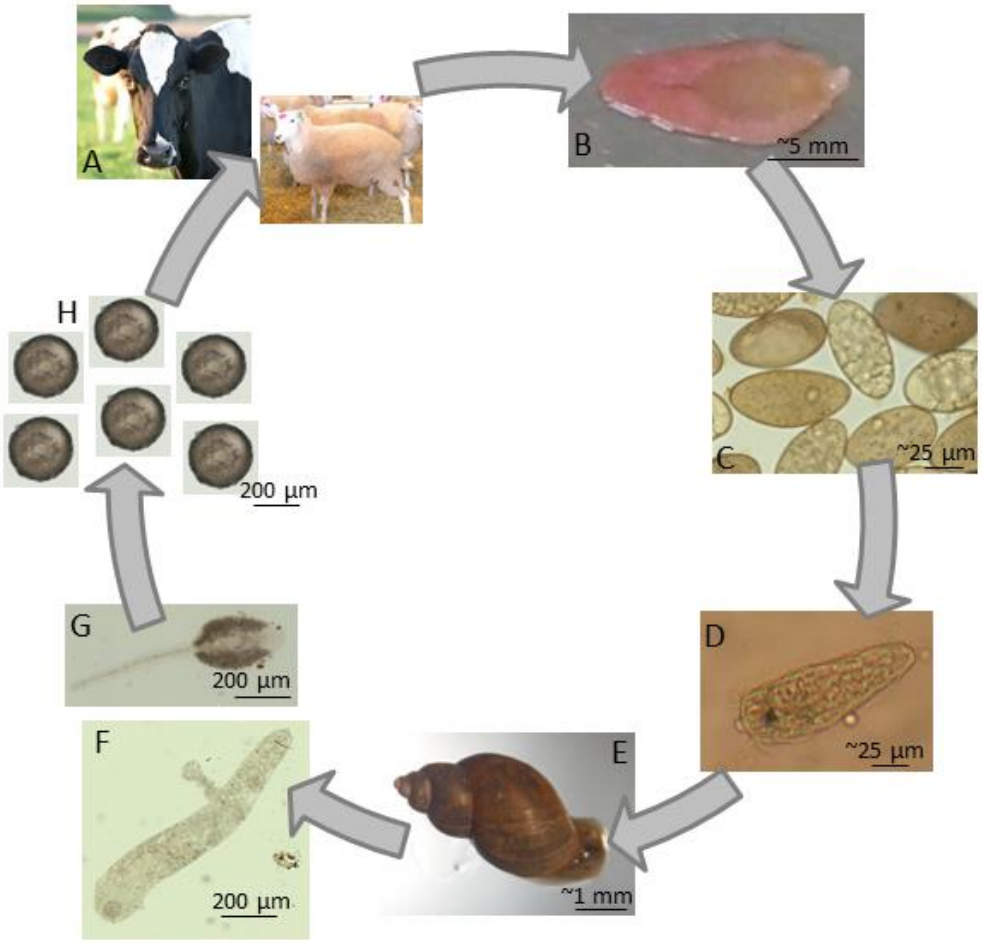

Figure 1.1: The life cycle of *Fasciola hepatica* in the UK; **A**: the definitive host (sheep and cattle; photos courtesy of D. J. L. Williams); **B**: adult *F. hepatica*; **C**: eggs of *F. hepatica*; **D**: the miracidium (photo courtesy of D. J. L. Williams); **E**: the intermediate host *Galba truncatula*; **F** and **G**: intra-molluscan life cycle stages: redia (**F**) and cercaria (**G**); **H**: metacercariae

1.2.2 Hatching of the miracidium from the egg and its infection of the snail intermediate host

The miracidium, inside the developed egg, is stimulated by light to rupture the operculum of the egg, which allows the miracidium to exit (Wilson, 1968). Miracidia are motile but short-lived life cycle stages that seek out and infect the snail intermediate host. The life span of a miracidium varies depending on water temperature from a mean of 6 hours at 25^{o}C to a mean of 35 hours at 6^{o}C (Smith and Grenfell, 1984).

The usual intermediate host in the UK is the mud snail *Galba truncatula.* The success of infection depends on the attractiveness of the snail to the parasite (Smith

and Crombie, 1982). Miracidia are attracted to chemicals produced by snails (Kalbe *et al.*, 1997), and mucus from the snail helps stimulate the attachment of the miracidium (Wilson *et al.*, 1971). The miracidium may penetrate the snail through the mantle, tentacles or foot. Penetration through the mantle provides the most favourable migration route to the preferred development sites (Préveraud-Sindou and Rondelaud, 1995). Differences in susceptibility to *F. hepatica* infection can exist within populations of snails of the same species, possibly due to variation in the immune response (Gutierrez *et al.*, 2003a; 2003b). Differences in the ability of a miracidium to infect a snail, measured by infection rate and snail survival rate, have also been noted when the miracidia have originated from different definitive hosts (Vignoles *et al.*, 2001; 2004). In naturally infected snails between 62 and 80 % were found to have been infected by one miracidium and infections with multiple miracidia accounted for the remaining infections (Rondelaud *et al.*, 2004).

1.2.3 Development of Fasciola hepatica *within* Galba truncatula

Once the miracidium has penetrated the snail it becomes the sporocyst form (Buzzell, 1983). Development of the parasite within the snail usually occurs in the renopericardial zone or in other areas of the mantel; if the sporocyst remains in the foot of the snail it is more likely to undergo limited, if any, development (Préveraud-Sindou and Rondelaud, 1995). As the parasite develops in the snail, epithelial necrosis and epithelial reconstitution with cellular hyperplasia, occurs in organs, including the digestive gland, albumen gland, gonads and kidney (Moukrim and Rondelaud, 1992). Infection with *F. hepatica* reduces the egg laying capacity of snails, although there is limited evidence that egg laying may be restored following shedding of cercariae (Dreyfuss *et al.*, 1999a).

The main feature of development in the snail is clonal expansion. The sporocyst is a ball of germinal cells each of which grow and divide, to produce multiple rediae (Thomas, 1883). These rediae exit the sporocyst and multiple cercariae, as well as other rediae, develop within them (Thomas, 1883). In natural infections up to four generations of rediae have been reported with the mean total number of rediae from single miracidium infections reported to be 15.8 (SD = 1.7; Rondelaud *et al.*, 2004). If a snail was naturally infected with multiple miracidia then the total number of rediae was higher (Rondelaud *et al.*, 2004; 2009).

Numerous experiments have explored the effects of infecting snails with different numbers of miracidia. The percentage of snails surviving to 30 days post infection (dpi) has consistently been shown to significantly decrease the more miracidia a snail is exposed to ($p < 0.001$; Dreyfuss *et al.*, 1999b; Vignoles *et al.*, 2010). The prevalence of infection with *F. hepatica* (usually reported as a percentage of those snails surviving to 30 dpi) has been reported to increase as the number of miracidia a snail is exposed to increases, but this change is not always significant (Dreyfuss *et al.*, 1999a; Vignoles *et al.*, 2010). It is hypothesised that the size of the snail is proportional to the number of miracidia the snail is able to support; and the better the nutrition the snail has access to the more miracidia the snail can support (Dreyfuss *et al.*, 1999b; Rondelaud *et al.*, 2004; Vignoles *et al.*, 2010). However all of these studies are based on the number of miracidia a snail is exposed to, rather than the number of miracidia that successfully infected the snail.

1.2.4 Shedding of cercariae from Galba truncatula

Experimental infections of snails have been used to characterise the conditions under which snails shed cercariae, as well as when this begins after exposure to miracidia, and how long snails shed for (Table 1.1).

Table 1.1: Summary of shedding of cercariae from *Galba truncatula*

Characteristic	Average	Range	Reference
Length of pre-patent period* (weeks)	7 – 8 (mode)	6 – 12	Dreyfuss *et al.*, 2006
Length of patent period^	17.2 to 47.2 days (mean)	1 – 11 weeks	Hodasi *et al.*, 1972; Vignoles *et al.*, 2003; 2015
Temperatures that stimulate snails to shed (^{o}C)	10 – 26		Kendall and McCullough, 1951
Natural light conditions that stimulate snails to shed (lux)	601 – 1200 (mode)	1 – > 3000	Vignoles *et al.*, 2014

* the pre-patent period begins at the point the snail is exposed to the miracidia and lasts until the snail starts to shed cercariae; ^ the patent period is the length of time the snail sheds cercariae

Temperature and light both affect the emergence of cercariae, and fresh water, including when there is flooding, also stimulates shedding of cercariae from snails (Kendall and McCullough, 1951). The length of time snails shed for is variable, and whilst snails can shed over time, they do not shed continuously. It has been noted that some snails shed at regular intervals of 6 to 8 days (Vignoles *et al.*, 2006). Output of cercariae is also highly variable; significant differences have been reported in the number of cercariae a snail produces in relation to the size, and the husbandry (diet and housing), of the snail. Whilst the number of miracidia a snail is exposed to has been shown to affect the percentage of snails surviving and the percentage of snails infected; no significant difference was noted in the number of cercariae produced (Figure 1.2). It should be remembered that these studies are usually based on experimental infections which may not mimic conditions found in the field.

1.2.5 Encystment of cercariae and survival of metacercariae

Once cercariae have been released from the snail they quickly encyst on substrates, for example vegetation, as metacercariae. The cercaria comes to rest, assumes a round form, produces a protective outer wall, and loses its tail (Thomas, 1883; Dixon, 1965). Studies on the survival of metacercariae show that temperature and relative humidity are critical factors. Metacercariae are resistant to freezing, only losing their viability after 12 hours at –20^{o}C. However, metacercariae are less resistant to desiccation, so at high temperatures (> 25^{o}C), or relative humidity levels of 75 to 80 %, infectivity decreases (Boray and Enigk, 1964; Boray, 1969). It is suggested that this susceptibility to desiccation may explain why metacercariae, assessed within two hours of emergence from snails, were more often found on the underside of leaves; this may be an adaptation that offers some protection from high temperatures (Hodasi, 1972). There is little other information available on the distribution of metacercariae over pasture, the spread of metacercariae from snail habitats, or at what height on grass the metacercariae encyst, which will change as grass grows.

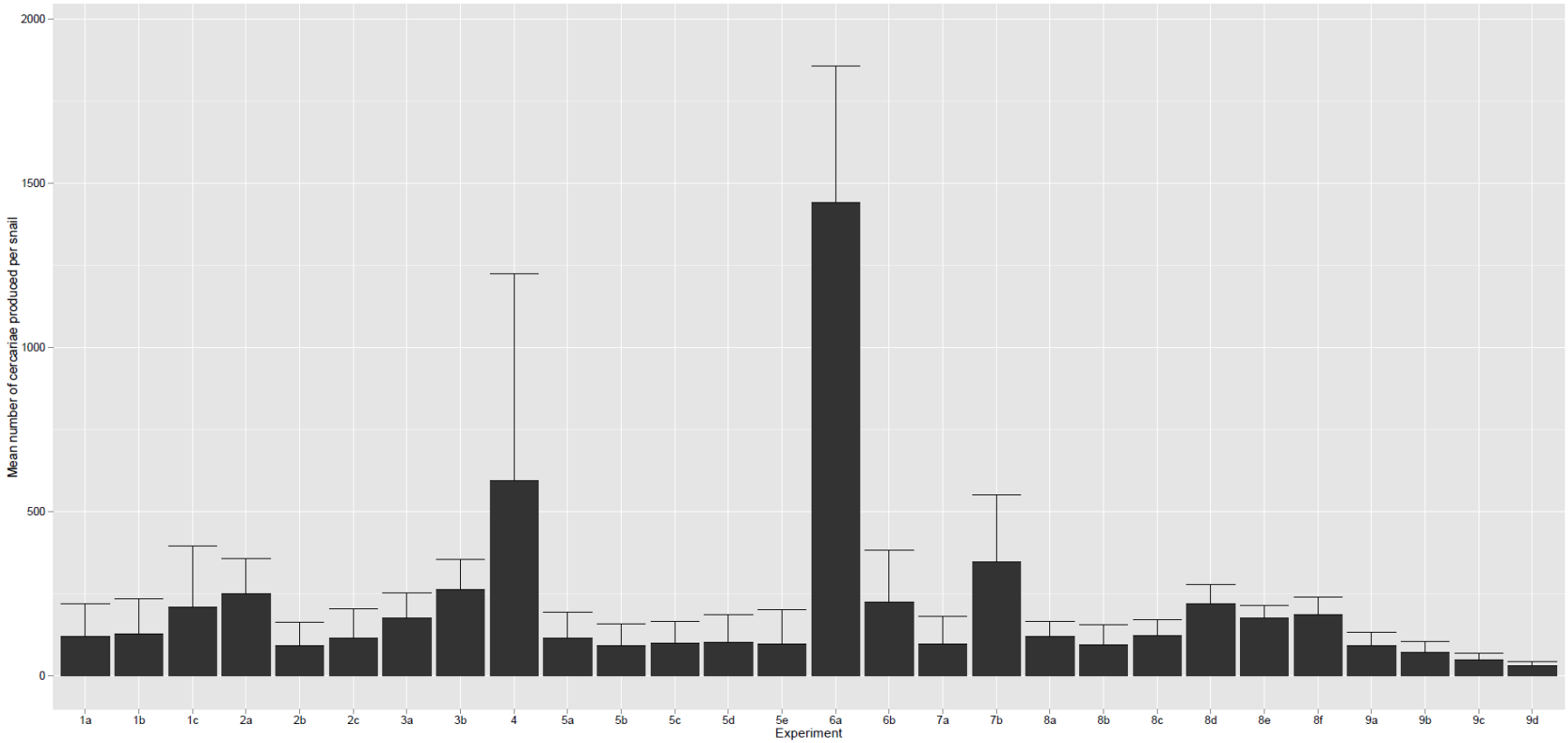

Figure 1.2: Reports of the mean number of cercariae shed per snail from experimental infections; error bar indicates standard deviation; **1a**, **b** and **c**: different populations of snails (Dreyfuss and Rondelaud, 1997); **2a**, **b** and **c**: different populations of snails (Vignoles *et al.*, 2003); **3a** and **b**: different populations of snails, no significant difference in cercarial output (Vignoles *et al.*, 2015); **4**: cercarial output of six different snails tracked weekly (Hodasi, 1972); **5a** to **5e**: snails exposed to 1, 2, 5, 10 or 20 miracidia, respectively, no significant difference in cercarial output (Dreyfuss *et al.*, 1999b); **6a**: well-fed snails had significantly higher cercarial output than **6b**: starved snails (Kendall and Ollerenshaw, 1963); **7a**: snails fed Cos lettuce had significantly lower cercarial output than **7b**: snails fed Cos lettuce with TetraPhyll (Belfaiza *et al.*, 2004); **8a to 8c**: snails kept in an aquarium had significantly lower cercarial outputs than **8d** to **8f**: snails kept in petri dishes (Rondelaud *et al.*, 2014a); **9a**: snails 7 mm in size had significantly different cercarial output compared to **9b** to **9d**: snails 6, 6 and 5 mm in size, respectively (Vignoles *et al.*, 2011)

1.2.6 Ingestion of metacercariae and development of adult parasites

The metacercariae are the infective stage and the definitive host becomes infected by ingesting metacercariae as it grazes. Once ingested, excystation occurs, which is an active process triggered by bile (Dixon, 1966). The early stages of infection have been studied most extensively in experimental infections of mice and rats. After excystation, juvenile parasites penetrate the intestine, usually through villi, and break down epithelial cells, connective tissue, and muscle fibres to pass into the abdominal cavity (Dawes, 1961a; 1963). In mice and rats, juvenile parasites reach the liver within 48 to 72 hours of infection (Dawes, 1961a; 1961b; 1963; Thorpe, 1965), whereas in sheep this is reported to occur within 90 hours (Sinclair, 1967). Once the juvenile parasites have passed through the liver capsule they begin to migrate through the liver, feeding and growing, until they reach the bile ducts (Urquhart, 1956; Dawes, 1961a; Thorpe 1965; Dow *et al.*, 1967; 1968). The time it takes for parasites to reach the bile ducts depends on the definitive host species; in mice and rats this can occur as early as four weeks after infection (Dawes, 1962; Thorpe 1965), in rabbits this can take five weeks (Urquhart, 1956), whilst in sheep this can take seven weeks (Dow *et al.*, 1968), and in cattle, between seven and eight weeks (Dow *et al.*, 1967). In cattle, *F. hepatica* has a predilection for the ventral lobe (Ross, 1965; Ross *et al.*, 1966), whilst in sheep the left lobe is preferred (Dow *et al.*, 1968). The age at which the parasites start to produce eggs also varies depending on the definitive host species. In mice this occurs between 35 and 39 days, in rabbits between eight and nine weeks, and in sheep and cattle around nine weeks (Urquhart, 1956; Dawes, 1962; Sinclair, 1967). In the absence of treatment, the adult parasites can be extremely long-lived; for example *F. hepatica* 11 years of age have been reported in sheep (Durbin, 1952).

1.3 Clinical signs, pathology and impact of infection on production

1.3.1 Clinical signs associated with Fasciola hepatica *infection*

Three clinical forms of disease are recognised in livestock acute, sub-acute and chronic; which form is seen depends on the number, and the period of time over which metacercariae are ingested. Acute disease is usually seen in sheep following infection with large numbers of metacercariae over a short period of time. Burdens of 1000 or more parasites are often reported; as the juvenile parasites migrate they

cause massive haemorrhage and liver damage. The only clinical sign that might be observed is sudden death due to acute haemorrhage. However other clinical signs may include weakness, pale mucous membranes, dyspnoea and ascites. The eggs of *F. hepatica* are absent from faeces, since disease occurs before the parasites reach maturity.

Sub-acute disease occurs following ingestion of a smaller number of metacercariae over a longer period of time. Burdens in sheep are usually around 800 to 1500 parasites, some of which will be mature adults residing in the bile ducts, so moderate to large numbers of eggs are found in the faeces. Clinical signs include a rapid loss of condition, anaemia, and submandibular oedema (bottle jaw) or ascites due to hypoalbuminaemia.

Chronic disease is caused following ingestion of smaller numbers of metacercariae, and disease is only seen once mature parasites accumulate in the bile ducts. Burdens in sheep are usually around 200 to 800 parasites. Clinical signs are anaemia, a gradual loss of condition, 'bottle jaw' and ascites. Moderate to high numbers of eggs are found in faeces (Sinclair 1962; 1967; Ross *et al.*, 1967; Boray, 1969; Reid *et al.*, 1970).

Calves are susceptible to all three forms of clinical disease with similar signs to those already described. Compared to sheep, higher numbers of metacercariae, particularly for acute and sub-acute disease, are required to produce the same clinical signs (Boray, 1969; Armour, 1975). This may be related to the calcium deposition that occurs within the bile ducts and associated hepatic fibrosis (Boray, 1969). Therefore disease in cattle is often subclinical, but is associated with lowered productivity (Armour, 1975).

1.3.2 Pathology associated with Fasciola hepatica *infection*

Infection with *F. hepatica* leads to cirrhosis of the liver (Ross, 1965; Dow *et al.*, 1968). As the parasites migrate through the liver they produce tracts, which can be seen grossly, and which are associated with haemorrhage and fibrosis. The inflammatory infiltrate seen varies depending on the age of the tract and the response of the definitive host. The inflammatory infiltrate consists of neutrophils, eosinophils, lymphocytes and macrophages. The inflammatory reaction associated with *F. hepatica* occurs earlier in sheep than cattle. The tracts eventually heal by granulation, and fibrin deposits may be observed on the external surface of the liver

(Urquhart, 1956; Dawes, 1961a; Thorpe 1965; Dow *et al.*, 1967; 1968). As *F. hepatica* is blood feeding, normochromic, haemorrhagic anaemia is commonly seen in all three clinical forms of disease (Dawes and Hughes, 1964; Ross *et al.*, 1966). This is accompanied by a significant increase in eosinophils (Ross *et al.*, 1966). As the juvenile parasites migrate across the liver they cause liver damage which leads to hypoalbuminaemia and an increase in liver enzymes (Ross *et al.*, 1967). Once the parasites become established in the bile ducts, the liver has a firm appearance, and the bile ducts are dilated with thickened walls. Cholangitis and bile duct hyperplasia are seen in reaction to the parasites within the bile ducts. In cattle calcium deposition occurs in the bile duct, a feature that is not seen in sheep (Dow *et al.*, 1967; 1968).

1.3.3 Production effects associated with Fasciola hepatica *infection*

Weight loss may be seen following infection with *F. hepatica* in sheep and cattle; this may be due to decreased dry matter intake as well as reduced food conversion efficiency (Hope Cawdery *et al.*, 1977; Hawkins and Morris, 1978). It has also been reported that even following treatment, weight gain in previously infected sheep was lower than in controls (Sinclair, 1962). Reductions in daily weight gains vary depending on parasite burdens, and are reported to range from 8.3 to 28 % in cattle (Hope Cawdery *et al.*, 1977) and 5.1 to 37.3 % in sheep (Hawkins and Morris, 1978). These studies were based on experimental infections; more recent work using information collected from an abattoir, and thus natural infections, found that infection with *F. hepatica* in beef cattle was associated with a decrease in mean warm carcass weight of 3.4 kg (0.7 %; Charlier *et al.*, 2009).

Recent studies have found that there is a decrease in milk yield associated with *F. hepatica* infection; reductions of between 0.7 and 3.9 kg per cow per day, are reported (Charlier *et al.*, 2007; 2012a; Mezo *et al.*, 2011; Howell *et al.*, 2015). In addition, treatment of dry cows with closantel has been found to increase milk yield (Charlier *et al.*, 2012b). This is in contrast to an older study which reported that infection with *F. hepatica* had no effect on milk production (Oakley *et al.*, 1979). However, the modern dairy cow is substantially higher yielding, so any stressors are likely to have more of an impact. Some studies have found no difference in milk butter fat or milk protein content (Mezo *et al.*, 2011; Howell *et al.*, 2015) whilst another study reported a significant decrease ($p < 0.001$) in milk butter fat of 0.06 % (Charlier *et al.*, 2012a). Finally, recent studies on the association between *F. hepatica*

and fertility in dairy cattle have either found no association or very small reductions in fertility (Charlier *et al.*, 2012a; Howell *et al.*, 2015).

1.4 Epidemiology of Fasciola hepatica *infection*

The epidemiology of *F. hepatica* infection is influenced by the population dynamics of the snail intermediate host, ambient temperature and moisture availability (Armour, 1975). Development of the egg, and of the parasite within the snail, is temperature dependent, with no development occurring below 10^{o}C. As the temperature increases so does the rate of development, up to approximately 25^{o}C (Boray, 1969; Ollerenshaw, 1971). Eggs that have not fully developed can overwinter on pasture, and development will continue the next year when temperatures increase; however these eggs probably only play a minor role in the overall epidemiology of the parasite (Ollerenshaw and Rowlands, 1959).

Miracidia are short lived and must seek out the snail intermediate host quickly. The pattern of disease varies depending on when during the year (*summer* or *winter*) the snail becomes infected. During the *summer infection,* snails are infected between May and June, the parasite develops within the snail and cercariae are produced from August to October causing contamination of pasture. In *winter infection,* snails are infected later in the year (late autumn) and when temperatures decrease, parasite development within the snail slows. The parasite overwinters in the snail and development increases when temperatures rise; cercariae are produced that contaminate pasture from May to July the following year (Ollerenshaw and Rowlands, 1959). The degree of wetness from May to October will determine the infection intensity for *summer infection*, whilst the weather in late summer, autumn, and early summer the next year, determine the intensity of *winter infection* and the overwintering potential (Ollerenshaw, 1971). In both Northern Ireland and Scotland it has been shown that *summer infection* predominates (Ross, 1970a; 1977), whilst studies in Wales showed differences in the levels of *summer* and *winter infection* depending on the prevailing weather conditions (Ollerenshaw and Rowlands, 1959). *Winter infection* may become more important as a result of climate change (Fox *et al.*, 2011); however it has been noted that experimental infections were more efficient in snails when performed in the spring or summer compared to the winter (Rondelaud and Dreyfuss, 1997). Snails can also become infected with more than

one species of parasite. One infection is usually dominant over the other (Rondelaud *et al.*, 2007a), and, therefore, co-infection in the snail can influence transmission of *F. hepatica*.

Ambient temperature also influences the release of cercariae from the snail. Cercariae are shed from the snail between the temperatures of 9 and 26^{o}C (Kendall and McCullough, 1951). Once, cercariae encyst as metacercariae, the survival of metacercariae depends on temperature and relative humidity (Boray and Enigk, 1964; Boray, 1969).

Ultimately these factors interact together to determine whether a population of snails is available for infection, whether these snails become infected, and whether the infection develops to produce metacercariae on pasture. Whether or not clinical disease is then seen will depend on the susceptibility of the definitive host, as well as the number of metacercariae they ingest, and the time period over which they are exposed to infective metacercariae.

1.5The definitive host: sheep and cattle farming in the UK

It is recognised that the husbandry and management of different farms have the potential to affect the population structure of parasites (Grillo *et al*., 2007). Husbandry and farming practices differ in the UK compared to other countries, and also across the UK, due to climatic and topographical factors. Animals within different production systems (breeding and / or milk production, meat production) will have different lifespans, may be raised in indoor and / or outdoor systems, and will be treated with anthelmintics at different times. All of these factors influence how long an animal is exposed to parasites on pasture, and how long parasites survive within the definitive host. There is also potential for *F. hepatica* within the definitive host to migrate around the country: either by farmers buying in replacement stock, or selling lambs and calves as "stores" for fattening. In some areas sheep and cattle may co-graze with each other, and may then be exposed to the same *F. hepatica* populations. The grazing patterns of sheep and cattle differ, which will influence how they are exposed to metacercariae on pasture. We do not definitively know whether sheep and cattle are permissive to infection by the same populations of *F.* hepatica, meaning that grazing the two species on the same pasture

increases exposure to the parasite as a whole, or whether there is evidence of genetic structuring of the parasite in the two definitive hosts.

1.6The snail intermediate host

1.6.1 Biology of the snail intermediate host Galba truncatula

Although *G. truncatula* are resistant to drought and frost (Schweizer *et al.*, 2007), temperatures around 18^{o}C are most favourable to their survival and development. When temperatures are below 10^{o}C their movement is restricted and they do not lay eggs (Heppleston, 1972); and if the environment is too dry they will aestivate for extensive periods (Ollerenshaw, 1959; Armour, 1975). In Spain, the highest number of snails was found in May, September and November (Manga-Gonzalez *et al.*, 1991). In Belgium, there was a peak of adult snails in July, and a peak of juvenile snails in October / November, both peaks coincided with periods of higher precipitation (Charlier *et al.*, 2014). In the Republic of Ireland, peaks in the abundance of *G. truncatula* were found in spring and autumn (Relf *et al.*, 2011). The typical life expectancy for a snail is around 10 to 12 months (Heppleston, 1972). The number of generations of snails per annum varies depending upon the climate (Armour, 1975), with one generation being most likely, but two generations can develop if the climate is suitable (Heppleston, 1972).

1.6.2 Methods for sampling wild snails

Galba truncatula is usually found in semi-aquatic habitats (Boray, 1969) including drainage furrows, slow moving streams, and river banks (Schweizer *et al.*, 2007; Rondelaud *et al.*, 2011). In the UK *G. truncatula* have been found in poorly drained, heavy clay or peat soils, as well as near to streams or natural springs (Crossland *et al.*, 1969; Crossland, 1976). Primary snail habitats are usually less than 5 m^2 (Rondelaud *et al.*, 2011), and support a relatively stable number of snails. However the number and size of temporary (secondary) habitats varies depending on the climate, and alters the carrying capacity from one year to the next (Crossland, 1976).

Sampling of snails is necessary to study population dynamics, as well as to determine the prevalence of *F. hepatica* infection within the snail. However, it can be difficult to identify snail habitats on farms. Sampling methods to collect snails

include searching a given quantity (e.g. one kilogram) of soil; however this only samples a small proportion of the habitat (Ollerenshaw, 1971). Snails may also be sampled by collecting all snails observed within a given time period. This method is biased towards the collection of larger snails and, since it is subjective, should be carried out by the same person each time (Heppleston, 1972). Finally snails may be sampled using a quadrant or transect method: collection of all the snails within a given area, for example 0.1 m^2, or along a given transect of a field (Heppleston, 1972; Charlier *et al.*, 2014). When comparing the timed and quadrant methods it was found that the quadrant method more accurately estimated snail distribution (Malone *et al.*, 1984/85), and in some instances a combination of these two methods has been used (Kaplan *et al.*, 1997).

1.6.3 Identification of wild caught snails

Accurate identification of snails is important because alongside *G. truncatula* many of the Lymnaeidae family are reported to sustain development of *F. hepatica* (Boray, 1969; 1978; Correa *et al.*, 2010). Such infections may be accidental, and may not always lead to the production of cercariae (Boray, 1969; 1978), but, if cercariae are produced, these infections will play a role in the transmission of *F. hepatica*. Some species of snail may only be susceptible to infection with *F. hepatica* early on in their development (Boray, 1978), and it is suggested that this age related resistance to *F. hepatica* infection was related to the maturation of the snail immune system (Dreyfuss *et al.*, 2000). A summary of the species of snails, other than *G. truncatula*, capable of supporting *F. hepatica* infection in Europe is shown in Table 1.2.

Lymnaeidae snails can be identified using morphological or molecular methods (Bargues and Mas-Coma, 2005). However definitive species identification of the Lymnaeidae family is problematic: there are reports of contradictory species identification using these two methods, and constant revisions to species definitions, along with the continued use of redundant nomenclature, confuses matters (Bargues *et al.*, 2001; Standley *et al.*, 2013; Dreyfuss *et al.*, 2014). For example *Lymnaea corvus, Lymnaea fuscus* and *Lymnaea palustris* were incorrectly grouped under the name *L. palustris* (Dreyfuss *et al.*, 2000); and the intermediate hosts responsible for *F. hepatica* transmission in Peru, *G. truncatula* and *Lymnaea neotropica*, were misclassified as *Lymnaea viator* using morphological methods (Bargues *et al.*, 2012).

Molecular work has improved our ability to identify species within the Lymnaeidae family (Standley *et al.*, 2013), and sequencing of the internal transcribed spacer (ITS) 2 has proved the most useful to date (Bargues and Mas-Coma, 2005). European Lymnaeidae snails show the clearest morphological differentiation between species compared to snails from other continents (Bargues and Mas-Coma, 1997). Analysis of the ITS-2 region of 65 individual European Lymnaeidae snails representing 13 species from 12 European countries by Bargues *et al.*, (2001) produced 31 different sequences. The length of the ITS-2 sequence differed from 370 to 491 bp depending on the species of snail. Sequence analysis of the ITS-2 region revealed that *G. truncatula* appeared highly homogeneous, whilst variation in the length of ITS-2 amongst *Lymnaea stagnalis*, *Stagnicola* spp. and *Omphiscola glabra* was due to different microsatellite repeat lengths (Bargues *et al.*, 2001). In the UK dichotomous keys are available to morphologically distinguish snails (Macan, 1977), and, to confirm this identification, the ITS-2 region can be amplified and sequenced (Relf *et al.*, 2011).

1.6.4 Detection of Fasciola hepatica *infection in snails*

Infection with *F. hepatica* can be detected by non-molecular methods: observing the shedding of cercariae, and crushing, or microscopic dissection, of the snail to visualise intra-molluscan parasite stages (Kaplan *et al.*, 1995). These methods have a number of disadvantages: both observation of shedding and dissection can be time consuming and dissection requires an experienced microscopist; the sensitivity of these methods can be poor in early stages of infection, and the intra-molluscan stages of different trematodes can be difficult to distinguish from one another giving poor specificity (Heussler *et al.*, 1993; Kaplan *et al.*, 1995; Caron *et al.*, 2008). It has been observed that the non-molecular methods described can underestimate the number of infected snails detected making them unsuitable for epidemiological studies (Kaplan *et al.*, 1997). A number of molecular techniques exist that have been used to identify *F. hepatica* DNA, including detecting *F. hepatica* infection in snails (Table 1.3). The majority of these are based on PCR. PCRs have generally been found to be more sensitive than microscopic methods (Caron *et al.*, 2007; Relf *et al.*, 2011), and PCRs that amplify regions of coding DNA are likely to give greater species specificity (Caron *et al.*, 2008). However the only method that gives an indication of the transmission potential of the

snail is to observe shedding of cercariae. PCRs detect infection with *F. hepatica* irrespective of whether snails have, or would have, shed cercariae. Snails can be infected with *F. hepatica* and not shed cercariae; in experimental infections the proportion of snails reported to be infected with *F. hepatica* but that do not shed cercariae ranges from 12.2 to 67.6 % (Dreyfuss *et al.*, 1999a; 1999b).

1.6.5 Prevalence of Fasciola hepatica *infection in the snail intermediate host*

Many of the studies that report prevalence of *F. hepatica* infection within the snail intermediate host only determine whether a snail is infected or not. Knowledge of whether the snail can shed cercariae, and information on the number of rediae and cercariae, are important to determine transmission potential (Ollerenshaw, 1971). The mean prevalence of *F. hepatica* in the intermediate host in the UK and the Republic of Ireland ranged from 3.1 to 16.7 %, and also varied depending on the time of year (Table 1.4). In France, large numbers of snails (5527, 7682, 11025, 18791, 19237) have been dissected to determine the prevalence of *F. hepatica* infection in *G. truncatula*. Prevalence of *F. hepatica* infection in the snail was usually around 5 % but ranged from 0.5 to 13.5 % at different times of the year (Rondelaud and Dreyfuss, 1997; Mage *et al.*, 2002; Rondelaud *et al.*, 2004; 2015).

Table 1.2: Species of snails, other than *Galba truncatula*, isolated from Europe that are capable of supporting *Fasciola hepatica* infection

Species of snail	Country	Type of Infection	Evidence of *F. hepatica* infection	Reference
Lymnaea fuscus	Sweden and France	Experimental	Infected juvenile snails (1 – 2 mm) shed cercariae	Dreyfuss *et al.*, 2000; Novobilsky *et al.*, 2013
Lymnaea palustris	Sweden	Natural	1 of 668 snails positive for *F. hepatica* ITS-2	Novobilsky *et al.*, 2013
		Experimental	Infected juvenile snails (1 – 2 mm) shed cercariae	
Omphiscola glabra	France	Natural	Dissection of > 15,000 snails showed prevalence of *F. hepatica* infection < 2.5 %; if *G. truncatula* is not present, then prevalence increases up to 14.5 %	Abrous *et al.*, 1999; Rondelaud *et al.*, 2001; Dreyfuss *et al.*, 2005
Potamopyrgus jenkinsi (antipodarum)	Wales	Natural	3 of 78 snails positive for *F. hepatica COX1* DNA	Jones *et al.*, 2015
Radix balthica (peregra)	Republic of Ireland	Natural	62 of 167 snails positive for *F. hepatica COX1* DNA	Relf *et al.*, 2009
	Belgium	Natural	7 of 4629 snails positive for *F. hepatica* 124 bp repeat	Caron *et al.*, 2014
	Wales	Natural	13 of 52 snails positive for *F. hepatica COX1* DNA	Jones *et al.*, 2015
Succinea spp.	Republic of Ireland	Natural	17 of 23 snails positive for *F. hepatica COX1* DNA	Relf *et al.*, 2009
Succinea putris	Sweden	Natural	1 of 42 snails positive for *F. hepatica* ITS-2 DNA	Novobilsky *et al.*, 2014

Table 1.3: Summary of the available molecular techniques to detect *Fasciola hepatica* DNA

Target DNA	Technique	Use	Sensitivity	Specificity	Reference
124 bp repeat (unknown location)	PCR	To determine if snails were infected with *F. hepatica*	Able to detect a snail infected with one miracidium within 90 minutes of exposure	Evaluated against *Fascioloides magna* and *Paramphistomum liorchis*	Kaplan *et al.*, 1995; 1997; Caron *et al.*, 2007
ITS-2	PCR	To determine if snails were infected with *F. hepatica*	Able to detect one experimentally infected snail within a pool of ten	Evaluated against *F. magna* and snails infected with *F. magna* and *Haplometra cylindracea*	Novobilsky *et al.*, 2013
86 bp repeat (unknown location)	Real time TaqMan PCR	To determine if snails were infected with *F. hepatica*	ND	ND	Schweizer *et al.*, 2007
ITS-2	PCR	To detect *F. hepatica* DNA in faeces from sheep	Able to detect *F. hepatica* in faeces 2 weeks post infection	Evaluated against *Teladorsagia circumcincta* and *Haemonchus contortus*	Robles-Perez *et al.*, 2013
RAPD of unknown location	PCR	To differentiate between *F. hepatica* and *Fasciola gigantica*	PCR was optimised using 10 – 50 ng *F. hepatica* DNA	Evaluated against a number of other parasites including: *Schistosoma* spp., *Paramphistomum cervi* and *F. magna*	McGarry *et al.*, 2007
Mitochondrial DNA	PCR	To differentiate between *F. hepatica* and *F. gigantica*	0.006 to 0.012 ng *F. hepatica* DNA	Evaluated against eight other platyhelminths including *Clonorchis sinensis* and *Opisthorcis viverrini*	Le *et al.*, 2012

ND = not determined; RAPD = Random amplified polymorphic DNA-PCR

Table 1.4: Summary of information on the prevalence of *Fasciola hepatica* infection within *Galba truncatula* in the UK and the Republic of Ireland

Country	No. of snails examined	Prevalence (%) of *F. hepatica* infection	Method used to detect *F. hepatica infection*	Reference
Wales	6000	> 16.7	Dissection	Kendall and Ollerenshaw, 1963; Ollerenshaw, 1971
Republic of Ireland	32	3.1	Dissection	Crossland *et al.*, 1969
England	2370	10.1 (range 4 – 21)*	Dissection	Crossland, 1976
Republic of Ireland	974	13.8 (range 3 – 25)*	PCR (*COX1* gene) and dissection	Relf *et al.*, 2011
Wales	134	13.4	PCR (*COX1* gene)	Jones *et al.*, 2015

*variation was seen in prevalence depending on the time of year

1.7 Control methods for Fasciola hepatica *infection*

The ideal way to control *F. hepatica* would be through an integrated approach and might involve (i) the use of anthelmintics to reduce parasite burden in the definitive host as well as pasture contamination with *F. hepatica* eggs, (ii) control of the snail population using molluscicides or biological control methods, and (iii) pasture management (Armour, 1975).

1.7.1 The use of anthelmintics to control Fasciola hepatica *infection*

The most common method of controlling *F. hepatica* is through the use of anthelmintics. There are a number of anthelmintics currently licensed in the UK for control of *F. hepatica*. These products include albendazole, clorsulon, closantel, nitroxynil, oxyclozanide and triclabendazole (NOAH Compendium, 2015; Norbrook 2015/2016). These drugs fall into four different chemical groups: halogenated phenols (nitroxynil), salicylanilides (closantel, oxyclozanide), benzimidazoles (albendazole, triclabendazole) and sulphonamides (clorsulon); and have different modes of action that include interference with energy metabolism via uncoupling of oxidative phosphorylation or inhibition of glycolysis, disruption of microtubule-

based processes, and induction of paralysis (Fairweather and Boray, 1999). The different drugs also have varying efficacy against different stages of *F. hepatica* (Fairweather and Boray, 1999); only triclabendazole is effective against juvenile parasites from two days (sheep) or two weeks (cattle) of age (Boray *et al.*, 1983; NOAH Compendium, 2015; Norbrook 2015/2016). Most of the licensed products either cannot be used in lactating animals, or have restrictions in place for their use around calving (NOAH Compendium, 2015; Norbrook 2015/2016). The shortest milk withdrawal periods for cattle are 60 and 72 hours for albendazole and oxyclozanide, respectively; but these drugs are only effective against mature parasites (> 12 weeks old), and albendazole must be used at double the normal dose rate (NOAH Compendium, 2015; Norbrook 2015/2016).

Triclabendazole is the most commonly used anthelmintic for treatment of fasciolosis because of its spectrum of activity against *F. hepatica* of all ages (Fairweather, 2005). Following administration, triclabendazole is metabolised by the liver to sulphoxide and sulphone metabolites. In sheep the flavin monooxygenase and cytochrome P450 enzyme systems have been shown to be responsible for this (Virkel *et al.*, 2006). It is suggested the mode of action of triclabendazole may be due to the combined effects of triclabendazole and its metabolites (Halferty *et al.*, 2009; Fairweather, 2011a). Although it has long been recognised that benzimidazole compounds disrupt microtubules (Lacey, 1988), the precise mode of action of triclabendazole is not known. In common with the other benzimidazoles, there is evidence of microtubule disruption in the tegument and reproductive tissues of *F. hepatica* following exposure to triclabendazole or its metabolites (Stitt and Fairweather, 1994; Hanna *et al.*, 2010). However, other effects have also been noted including interference with energy metabolism via uncoupling of oxidative phosphorylation, and inhibition of protein synthesis (Carr *et al.*, 1993; Stitt *et al.*, 1995). Despite the recognition that the mode of action of triclabendazole is likely to be due to the combined effects of triclabendazole and its metabolites, the majority of research into the mode of action has focused on the effect of the sulphoxide metabolites only.

Regular anthelmintic treatments have been suggested as a control strategy to reduce *F. hepatica* prevalence in both sheep and cattle. If successful the frequency of treatments can be reduced once pasture contamination is controlled (Fawcett, 1990; Parr and Gray, 2000). However complete eradication of *F. hepatica* on a farm is

unlikely due to the movement of stock, and reservoirs of infection in wild definitive hosts, such as rabbits (Parr and Gray, 2000). On sheep farms this practice of regular anthelmintic treatments may continue, and treatments may be even more frequent to stop juvenile parasites from establishing and help prevent outbreaks of acute fasciolosis. However, treatment of livestock is problematic due to the meat and milk withdrawal periods. Since cattle are not usually grazed all year round (compared to sheep) cattle are in general treated less frequently than sheep. The overreliance on triclabendazole, due to its efficacy against juvenile parasites, for treatment of sheep and cattle has resulted in the development of resistance to this drug (Overend and Bowen, 1995).

1.7.2 Disease forecasting and spatial models

Climate is a limiting factor for many of the stages of development of *F. hepatica*. The first method of disease forecasting for fasciolosis, developed by Ollerenshaw and Rowlands, (1959) was based on the number of days it rains, the amount of precipitation, and evapotranspiration. This method was first applied in Anglesey, then validated in England and Wales (Ollerenshaw, 1966), and is still the basis for disease forecasting in the UK by the National Animal Disease Information Service (NADIS). These forecasts assist farmers when planning parasite control (NADIS, 2016). However, due to the complex interaction between the parasite and the environment, methods developed in one country cannot necessarily be directly applied to other areas (Manga-Gonzalez *et al.*, 1991). A number of studies have attempted to refine the Ollerenshaw and Rowlands, (1959) method by using different combinations of meteorological data (Ross, 1970b; Gettinby *et al.*, 1974; Malone *et al.*, 1987; McIlroy *et al.*, 1990). However these models have not achieved accurate predictions at a local level, due to the highly localised nature of snail habitats (Malone *et al.*, 1992). More recently, models have been used to identify environmental and farm management risk factors for *F. hepatica* infection. In addition to meteorological and environmental factors, pasture management factors, such as mowing of pastures and the length of the grazing season, have also been found to be important. The most successful model was able to explain 85 % of the observed variation in *F. hepatica* infection, but included information about snail habitats on individual farms. It is difficult to predict the risk of fasciolosis at the

individual farm level without this detailed characterisation of snail habitats (McCann *et al.*, 2010b; Bennema *et al.*, 2011; Charlier *et al.*, 2011; 2014; Howell *et al.*, 2015).

1.7.3 Other methods of controlling Fasciola hepatica *infection: controlling the snail population, pasture management and vaccines*

Molluscicides are not licensed in the UK due to their adverse environmental effects. However extensive research was conducted in the 1960s and 1970s on the use of molluscicides as a control measure for fasciolosis. The molluscicides, copper pentachlorphenate and N-tritylmorpholine, were found to decrease numbers of snails, as well as reduce the burden of parasites within definitive hosts, although some transmission of the parasite usually remained. The extent to which these effects were seen was dependent on the timing and frequency of molluscicide application; eventually the snail population recovered. Therefore, if molluscicides were to be used, the timing of their application would need to be based on climatic conditions (Gordon *et al.*, 1959; Ollerenshaw, 1966, Ross and Taylor, 1968; Crossland *et al.*, 1969; 1977; Ross *et al.*, 1969; Urquhart *et al.*, 1970; Crossland, 1976).

Biological control methods, including the use of predators such as *Sepedon fuscipennis*, against snail populations have also been suggested as a method of controlling *F. hepatica* infection (Berg, 1953; Gordon and Boray, 1970; Whitelaw and Fawcett, 1982). The use of predators (*Procambarus clarkii* – crayfish and *Macrobrachium* spp. – prawns) against the intermediate hosts of human schistosomes has been explored. These predators have been shown to decrease snail numbers and in some instances reduce the likelihood of infection in the human population. However further research on the ecological impacts, as well as the implementation of these control methods on a larger scale and longer term, are needed (Mkoji *et al.*, 1999; Savaya Alkalay *et al.*, 2014; Sokolow *et al.*, 2014).

Fields which contain suitable habitats for the snail pose a risk to grazing animals. Therefore it is suggested that these pastures should be avoided and snail habitats fenced off (Armour, 1975), or drained (Crossland, 1976). However these management practices are not usually used as the sole control method (Osborne, 1967).

There is also much interest in the development of a vaccine for *F. hepatica*, although a vaccine that is commercially viable is not yet available. Antigens that have been trialled as vaccine candidates include fatty acid binding protein, cathepsin

proteases, glutathione s-transferases, peroxiredoxin, and haemoglobin, as well as combinations of these antigens. These trials have produced mixed results but essentially have not been able to induce a consistent protective immune response (Hillyer *et al.*, 1987; Sexton *et al.*, 1990; Dalton *et al.*, 1996; Golden *et al.*, 2010; Toet *et al.*, 2014). Some antigens have shown no significant protection; whilst others have reduced parasite burdens, but levels of protection are variable (ranging from 11 to 98.5 %; reviewed by Toet *et al.*, 2014), or are only seen in some animals. The immune system of the definitive host is modulated, by *F. hepatica*, towards a Th2 and regulatory response; however it is known that a Th1 response is needed for protection (Dalton *et al.*, 2013; McNeilly and Nisbet, 2014; Toet *et al.*, 2014).

The most successful vaccine candidate to date is cathepsin L1. This antigen, given in Montanide^{TM} ISA 70VG or ISA 206VG adjuvants, stimulated a Th1 response, and reduced parasite burdens by 48.2 % in a field challenge experiment (Golden *et al.*, 2010). Cathepsin L1 is essential to many of the functions of *F. hepatica* including the emergence of the parasite from metacercariae, migration, and acquisition of nutrients. Current research is based around producing a protective immune response through choice of adjuvant as well as antigen. In addition cathepsin L1 is one of the molecules responsible for immunomodulation by *F. hepatica*. A vaccine targeting this molecule would have an additional advantage: if an animal is challenged following vaccination, this immunomodulation would be disturbed, and the parasite would be exposed to the protective immune response of the definitive host (Dalton *et al.*, 2013; McNeilly and Nisbet, 2014; Toet *et al.*, 2014).

1.8 Anthelmintic resistance

1.8.1 Reports of Fasciola hepatica that are *resistant to triclabendazole*

Resistance of *F. hepatica* to triclabendazole was first reported in Australia in 1995 (Overend and Bowen, 1995). Since then triclabendazole resistance has been reported in *F. hepatica* from sheep and cattle within the UK (Mitchell *et al.*, 1998; Thomas *et al.*, 2000; Daniel and Mitchell, 2002; Sargison *et al.*, 2010; Sargison and Scott, 2011; Daniel *et al.*, 2012; Gordon *et al.*, 2012; Hanna *et al.*, 2015), and in a number of other countries including Argentina, the Republic of Ireland, the Netherlands, Spain and Peru (Moll *et al.*, 2000; Gaasenbeek *et al.*, 2001; Álvarez-Sánchez *et al.*, 2006; Mooney *et al.*, 2009; Olaechea *et al.*, 2011; Ortiz *et al.*, 2013).

Recently in Wales and England, resistance to triclabendazole was detected on seven of 25 sheep farms studied. This gives a baseline estimate of the prevalence of triclabendazole resistance (Daniel *et al.*, 2012).

It has been suggested that some of the reports of triclabendazole resistance are anecdotal, and may instead be treatment failure due to, for example, incorrect dosing or variable drug quality (Fairweather, 2011a). Some of the studies investigating resistance to triclabendazole have taken steps to ensure under dosing or drug quality are not confounding factors by performing efficacy testing. Furthermore suspected triclabendazole-resistant *F. hepatica* were collected from sheep at slaughter, propagated through snails in the laboratory, and the metacercariae produced were used to infect sheep. Following treatment with triclabendazole, parasites remained at post mortem; thereby confirming resistance using a dose and slaughter trial (Overend and Bowen, 1995; Gordon *et al.*, 2012). The majority of reports diagnose resistance to triclabendazole using faecal egg count reduction tests (FECRT; Overend and Bowen, 1995; Moll *et al.*, 2000; Gaasenbeek *et al.*, 2001; Álvarez-Sánchez *et al.*, 2006; Mooney *et al.*, 2009; Olaechea *et al.*, 2011; Sargison and Scott, 2011; Daniel *et al.*, 2012; Gordon *et al.*, 2012; Ortiz *et al.*, 2013; Hanna *et al.*, 2015). FECRT have recognised limitations for diagnosing resistance to triclabendazole: faecal egg counts only detect patent infections, and *F. hepatica* eggs may be shed intermittently or stored in the gall bladder after death of adult parasites (Coles *et al.*, 2006; Fairweather, 2011a). For these reasons further tests (*in vitro* efficacy tests, histology, or comparative anthelmintic efficacy testing) have been performed alongside FECRT to confirm resistance to triclabendazole, and help to validate the use of the FECRT as a diagnostic tool (Overend and Bowen, 1995; Álvarez-Sánchez *et al.*, 2006; Daniel *et al.*, 2012; Gordon *et al.*, 2012; Sargison, 2012; Ortiz *et al.*, 2013; Hanna *et al.*, 2015). In Spain, there are reports of *F. hepatica* that are resistant to triclabendazole and clorsulon and / or albendazole (Álvarez-Sánchez *et al.*, 2006; Vara-Del Río *et al.*, 2007). The evolution of multidrug resistant parasites has implications for veterinarians and farmers when designing disease control programs.

The treatment of choice for human fasciolosis is also triclabendazole (WHO, 2007). There are reports of treatment failure, and, by inference, resistance to triclabendazole in human cases in Chile, the Netherlands and Turkey (Saba *et al.*, 2004; Winkelhagen *et al.*, 2012; Gil *et al.*, 2014). Given the role that animals play in

the transmission of fasciolosis to humans, resistance is probably more common than reported in areas where fasciolosis is endemic in humans and animals. Although there is debate about how widespread triclabendazole resistance may be it is considered to be a substantial threat to the current and future control of *F. hepatica*.

1.8.2 Mechanisms of triclabendazole resistance in Fasciola hepatica

The mechanism of triclabendazole resistance remains unknown despite extensive research and the availability of a number of laboratory isolates of *F. hepatica* with known resistance statuses (reviewed by Hodgkinson *et al.*, 2013). Defining the mechanism of resistance is further complicated because the exact mode of action of triclabendazole is not known (see 1.7.1). Microtubule disruption is seen in *F. hepatica* following exposure to triclabendazole (Stitt and Fairweather, 1994; Hanna *et al.*, 2010), and mutations within β-tubulin have been implicated in benzimidazole resistance of a number of nematodes. Therefore mutations within the tubulin genes were considered as a possible mechanism of resistance. However, no amino acid polymorphisms were found at proposed benzimidazole binding sites in both triclabendazole-susceptible and triclabendazole-resistant *F. hepatica* (Robinson *et al.*, 2001; 2002; Ryan *et al.*, 2008). Comparison between *F. hepatica* isolates, with different anthelmintic resistance statuses, collected from sheep in North West Spain did reveal a single nucleotide polymorphism (SNP) in the 28S rDNA (Vara-Del Río *et al.*, 2007), but the significance of this in relation to drug resistance remains unknown.

There is a greater body of evidence that uptake, efflux and metabolism of triclabendazole may be altered within resistant parasites. Significantly ($p < 0.05$) lower levels of triclabendazole and triclabendazole sulphoxide were found within triclabendazole-resistant *F. hepatica* than triclabendazole-susceptible parasites (Alvarez *et al.*, 2005; Mottier *et al.*, 2006). This may be due to reduced uptake by diffusion across the tegument, or increased efflux of triclabendazole. There is evidence that the transmembrane transporter P-glycoprotein may be involved in increasing the efflux of triclabendazole from the parasite (Alvarez *et al.*, 2005; Mottier *et al.*, 2006). This is supported by the discovery of a variant P-glycoprotein that was found at higher frequency in resistant parasites than susceptible parasites (Wilkinson *et al.*, 2012). However, the significance of this is not known given that only a small number of parasites were examined in this study (Wilkinson *et al.*,

2012), and the same SNP was not present in Australian populations of triclabendazole-resistant *F. hepatica* (Elliott and Spithill, 2014).

Fasciola hepatica is itself able to metabolise triclabendazole to its sulphoxide and, relatively inert, sulphone metabolites. As in sheep, the main metabolic pathway for metabolism of triclabendazole to its sulphoxide is the flavin monooxygenase enzyme system, and of the sulphoxide to the sulphone is the cytochrome P450 enzyme system. Parasites resistant to triclabendazole show increased metabolism of triclabendazole to its sulphoxide and then sulphone metabolites (Robinson *et al.*, 2004; Alvarez *et al.*, 2005). Increased metabolism of triclabendazole as a mechanism of resistance is supported by the potentiation of triclabendazole when used in combination with either ketoconazole or methimazole. These two chemicals inhibit the cytochrome P450 and flavin monooxygenase enzyme systems, respectively. The majority of this work is *in vitro* but *in vivo* experiments in rats show similar findings (Devine *et al*., 2009; 2010a; 2010b; 2011a; 2011b; 2012). It is also possible that a combination of mechanisms may be responsible for conferring triclabendazole resistance (Brennan *et al.*, 2007).

At the University of Liverpool the genetic basis of triclabendazole resistance is being investigated using a classical genetic cross of triclabendazole-sensitive and -resistant parasites with subsequent genome-wide mapping of resistance loci to complement those studies exploring specific mutations or mechanisms in candidate genes (Hodgkinson *et al.*, 2013). Whatever gene(s) or pathways are involved in triclabendazole resistance, understanding genetic variation and gene flow in parasite populations as a whole is essential to facilitate our understanding of the genetic evolution and spread of anthelmintic resistance (Gilleard and Beech, 2007).

1.9 Current understanding of **Fasciola hepatica** *genetics*

1.9.1 The ploidy of Fasciola hepatica

In the UK, *F. hepatica* was first reported to be diploid with 10 bivalent chromosomes (2n = 2x = 20) by Sanderson (1953). Since then there have been several reports describing fluke with 10 bivalent pairs of chromosomes including the triclabendazole-resistant laboratory isolate of *F. hepatica* Sligo (n = 5) originally isolated from sheep in the Republic of Ireland (Fletcher *et al.*, 2004), wild-type *F. hepatica* (n = 15) from cattle livers in Northern Ireland (Fletcher *et al.*, 2004) and *F.*

hepatica (n = 10) obtained from a cattle liver in Slovakia (Reblánová *et al.*, 2011). In contrast, the triclabendazole-susceptible laboratory isolate Cullompton (n = 10), originally isolated from sheep in the UK, was shown to be triploid with 30 univalent chromosomes (2n = 3x = 30; Fletcher *et al.,* 2004). The triploid Cullompton parasites were aspermic due to a failure of the first meiotic division during spermatogenesis (Fletcher *et al.*, 2004), and were therefore assumed to undergo parthenogenesis in order to reproduce (Hanna *et al.*, 2008).

In Asia, where *F. hepatica* and *F. gigantica* exist in combination, *Fasciola* spp. are reported to be diploid, triploid, or mixoploid (mixoploid parasites have a mixture of cells, some with a diploid number of chromosomes and some with a triploid number; Rhee *et al.*, 1987; Terasaki *et al.*, 1998; 2000; Itagaki *et al.*, 1998; 2005a; 2005b; 2009). In the UK *F. hepatica* is the only species that occurs; we do not know the extent to which diploid and triploid parasites are found in natural infections, yet knowledge of ploidy is important for a number of downstream applications, particularly population genetic analyses.

1.9.2 Current knowledge of Fasciola hepatica *population genetics and genetic markers available for these studies*

Random amplified polymorphic DNA-PCR (RAPD) has been used to differentiate *F. hepatica* and / or *F. gigantica* and in some instances has also found differences in the RAPD patterns of *F. hepatica* from different mammalian hosts (Vargas *et al.*, 2003; Ramadan and Saber, 2004; Aldemir, 2006; McGarry *et al.*, 2007). However, the use of RAPD analysis is cautioned against for parsimony analyses (Backeljau *et al.*, 1995) and over reproducibility of the banding patterns (Ellsworth *et al.*, 1993). Sequence-related amplified polymorphism (SRAP) is a genetic marker system developed by Li and Quiros, (2001) that has been used to analyse adult *F. hepatica* (n = 483) from six different mammalian hosts from a variety of geographical locations in Spain (Alasaad *et al.*, 2008). Whilst the markers were polymorphic, the level of intraspecific variation between *F. hepatica* from different host species and geographical locations was low (Alassad *et al.*, 2008). Although SRAP has been recognised as a simple, reliable genetic marker system (Li and Quiros, 2001), the population genetic analyses possible from this system are considered to be limited (Vázquez-Prieto *et al.*, 2011). Protein electrophoresis, used to study the variation of allozymes in *F. hepatica* (n = 110) from three cows each

from a different farm in North West Spain, suggested a panmictic population of parasites with high genetic variability, low population structure and high gene flow (Vázquez-Prieto *et al.*, 2011). However the use of allozymes to determine the extent of genetic differentiation has been questioned (Beveridge, 1998).

1.9.2.1 Ribosomal and mitochondrial DNA markers

Ribosomal and mitochondrial DNA (rDNA and mtDNA) have been used to investigate the origins of triploidy in *Fasciola* spp. (see Chapter 2). They are also the most extensively applied genetic markers to study genetic variation in *Fasciola* spp. by assessing sequence variation. Examination of the 28S rDNA of *F. hepatica* and *F. gigantica* revealed no intraspecific variation and only a few interspecific polymorphisms (Marcilla *et al.*, unpublished cited in Marcilla *et al.*, 2002). The ITS-1 (Ichikawa and Itagaki, 2010) and the ITS-2 (Itagaki and Tsutsumi, 1998; Ali *et al.*, 2008; Erensoy *et al.*, 2009) sequences of rDNA have been used to discriminate between *F. hepatica*, *F. gigantica* and intermediate forms of *Fasciola* spp. However, the evidence indicates these markers have low levels of intraspecific variation and would therefore be of limited use for population genetic studies.

The complete mitochondrial genome of *F. hepatica* was sequenced from the Geelong strain in Australia (14,462 bp; Accession Number: AF216697). When this was compared to a fragment of mtDNA from a strain from Salt Lake City, USA, there was < 1 % intraspecific variation, which equated to 28 nucleotide differences and four amino acid changes in the cytochrome c oxidase 1 (*COX1*) gene, and two amino acid changes in both the NAD(P)H dehydrogenase (*NAD*)*1* and *NAD3* genes (Le *et al.*, 2001). Further analysis of Australian *F. hepatica* isolates (n = 208) found 18 haplotypes for the *NAD1* gene and six for the *COX1* gene, with 2.6 % and 1.4 % polymorphic sites, respectively (Elliott *et al.*, 2014). Similar proportions of polymorphic sites in the *NAD1* and *COX1* genes were reported in *F. hepatica* from eastern Europe and western Asia (Morozova *et al.*, 2004; Semyenova *et al.*, 2006). In addition little genetic structure was found between the isolates of *F. hepatica* from eastern Europe compared to those from western Asia, possibly indicating high gene flow between these parasite populations (Semyenova *et al.*, 2006). In contrast detection of SNPs in the 28S rDNA and mtDNA did identify a distinct difference between north-eastern and south-eastern European *F. hepatica* isolates. However, no genetic structuring was identified from the same isolates when the *β-tubulin 3* gene

was assessed (Teofanova *et al.*, 2011). The 28S rDNA is reported to be highly conserved in *F. hepatica* (Mas-Coma *et al.*, 2009) which limits its usefulness for population genetic studies. A number of the studies that make use of mitochondrial markers use only short sequences of the *NAD1* and *COX1*, which makes comparative analysis between studies difficult or impossible (Mas-Coma *et al.*, 2009).

Analysis of European *F. hepatica* (n = 221) using a restriction fragment length polymorphism PCR (RFLP-PCR) at three loci on the mitochondrial genome revealed 52 haplotypes. Forty five parasites genotyped from one calf, that had grazed the same pasture for one season, were found to have ten different haplotypes; whilst all parasites genotyped from the Fairhurst isolate (a triclabendazole-susceptible lab isolate) were the same haplotype (Walker *et al.*, 2007). The same RFLP-PCR was used to compare *F. hepatica* (n = 422) from two cattle farms in the Netherlands and revealed 38 and 56 haplotypes from each population. Temporal analysis was performed at one farm and showed that the haplotypes present changed significantly from one year to the next (Walker *et al.*, 2011). Although cattle were born on this farm, sheep, brought in from other areas, grazed on the same pastures; so may have introduced novel parasites. Overall low population structure was found between these isolates as F_{ST} was 0.0228 (Walker *et al.*, 2011). These studies have shown high diversity of mitochondrial markers; however there are limitations to using these markers for studying population diversity. Mitochondrial DNA shows only maternal inheritance, and these markers may not be neutral as they can be influenced by selection (Galtier *et al.*, 2009).

1.9.2.2 Microsatellites

Microsatellites offer a number of advantages over the other genetic markers already discussed. They have been found to be randomly distributed in mammalian DNA (Stallings *et al.*, 1991), and in closely related species positions of microsatellites are conserved allowing comparison with the same sets of primers (Moore *et al.*, 1991). Microsatellites evolve neutrally (Ellegren, 2004); they are inherited from both parents so offer a more comprehensive approach to the study of gene flow (Johnson *et al.*, 2006), and since they have a high mutation rate they tend to be polymorphic markers (Abdelkrim *et al.*, 2009). Therefore microsatellites are considered to be a more powerful tool than allozyme analysis, RAPD markers and mtDNA markers (Laoprom *et al.*, 2010).

Five polymorphic microsatellite markers were isolated from Bolivian *F. hepatica* (Hutrez-Boussès *et al.*, 2004) and have been used on a limited basis to assess the genetic diversity of 142 adult *F. hepatica* and *F. gigantica* in Egypt (Dar *et al.*, 2011a) as well as to differentiate between the two species (Dar *et al.*, 2013). More recently a panel of 15 polymorphic microsatellites has been developed from the UK *F. hepatica* population (Cwiklinski *et al.*, 2015a). The largest study to date to assess the population structure of *F. hepatica* used a combination of microsatellites and allozymes (Vilas *et al.*, 2012). The study, performed in Spain, analysed a total of 587 adult *F. hepatica* from the livers of 10 cattle and 10 sheep. Genotypic diversity was on average 0.577 in parasites from sheep and 0.747 in parasites from cattle, repeated multilocus genotypes (MLGs) were observed in sheep and cattle with significant P_{sex} values at n = 2 or n = 3 (Vilas *et al.*, 2012). Standardised F_{ST} values were 0.087 and 0.170 in cattle and sheep, respectively excluding the parasites with repeated MLGs (Vilas *et al.*, 2012). However, all 10 sheep were from the same farm, and all 10 cows were from different farms. This makes it difficult to compare the population genetic structure between different farms, and makes comparisons between the two definitive host species difficult.

1.9.2.3 The genome of Fasciola hepatica

A draft genome for *F. hepatica* has recently been published. The genome is 1.3 Gb, and, despite only five isolates of *F. hepatica* being used, shows a high level of polymorphism: 48 % of genes had at least one non-synonymous SNP present, and the average non-synonymous nucleotide diversity across the coding sequence was 5.2×10^{-4}. These values are based on genome wide re-sequencing and are calculated based on a large number of genes (22, 676 gene models are supported by RNAseq data; Cwiklinski *et al.*, 2015b).

1.9.3 Comparative population genetics of other important parasites of livestock and humans

When studied within a single country many nematode species of livestock are panmictic (Anderson *et al.*, 1998). When microsatellites have been used to perform population genetic studies there is evidence of genetic differentiation between parasites from different continents including in *Teladorsagia circumcincta* and *Haemonchus contortus* (Grillo *et al.*, 2007; Redman *et al.*, 2008). Conversely isolates

of *H. contortus* from the UK, separated only by short distances, also showed genetic differentiation between isolates (Gilleard, 2013).

High genetic diversity and high gene flow is reported in a number of parasites of livestock including *Teladorsagia* spp. and *H. contortus* from sheep, and *Ostertagia ostertagi* and *Haemonchus placei* from cattle. High levels of gene flow are thought to be maintained, in part, by movement of the definitive host. Most of the genetic diversity seen exists between individual parasites within the definitive host. This may be due to the large parasite burdens, and thus large effective population sizes, associated with these parasites (Blouin *et al.* 1995; Anderson *et al.*, 1998; Braisher *et al*., 2004; Grillo *et al.*, 2007). Whilst some of these studies were based on mtDNA markers (Blouin *et al.*, 1995; Braisher *et al.*, 2004) similar findings were found when using microsatellites (Grillo *et al.*, 2007) which are considered to be a more robust marker.

Due to the public health significance much research has been conducted into the population genetic structure of *Schistosoma* spp. These trematode parasites differ from *Fasciola* spp. as the sexes are separate. This, along with the differing life cycle and epidemiology compared to *F. hepatica*, has the potential to influence the population structure and genetic diversity seen which is an important consideration when drawing comparisons. Numerous studies using microsatellite markers have been performed in *Schistosoma* spp. High genetic diversity is usually reported in *Schistosoma haematobium, Schistosoma japonicum* and *Schistosoma mansoni*. However, compared to other African countries a lower heterozygosity was reported in *S. haematobium* from South Africa. The significance of this is unknown as only a small number of parasites (12) were assessed (Wang *et al.*, 2006; Glenn *et al.*, 2013; Gower *et al.*, 2013). High genetic differentiation is reported between *S. haematobium* and *S. mansoni* from different countries whilst high gene flow is seen between those parasites from the same regions or countries (Gower *et al.*, 2011; 2013). Interestingly differences are noted in the level of genetic diversity of parasites from different species of definitive host in *S. japonicum* (Wang *et al.*, 2006). The availability of genomes for *Schistosoma* spp. along with robust, multilocus, multiplex genotyping systems using microsatellite loci will further advance the population genetic analyses possible for these parasites (Webster *et al.*, 2015).

1.10 Aims and objectives

The parasite *F. hepatica* is an important cause of disease in sheep and cattle in the UK, and its prevalence is increasing. Resistance to triclabendazole, a product that is used widely to control *F. hepatica* infection in sheep and cattle, has been reported, and is common in some regions (Daniel *et al.*, 2012). To understand how resistance spreads through *F. hepatica* populations, a better understanding of genetic variation and how genes flow through populations is required. Therefore the aim of this project was to determine the ploidy (Chapter 2) and population genetic structure of *F. hepatica* infecting sheep and cattle in Great Britain (Chapter 3), and define how passage of the parasite through the snail intermediate host may influence genetic diversity (Chapter 4).

In Chapter 2 the proportion of *F. hepatica* that are diploid or triploid was determined. In Chapter 3 a multiplex PCR method to genotype parasites with our validated panel of microsatellite markers (Cwiklinski *et al.*, 2015a) was optimised. This panel was used to genotype a representative sample of adult parasites collected from sheep and cattle at slaughter to determine (a) the genetic diversity in the British *F. hepatica* population, (b) whether parasites of the same MLG are found within the definitive host, and (c) evaluate the population structure of British *F. hepatica*. In Chapter 4 the role of the intermediate host was explored by establishing whether a snail can be infected with, and produce cercariae of, more than one genotype; and evaluating the population genetic structure of *F. hepatica* in field populations of *G. truncatula.*

CHAPTER 2

Determining the ploidy of *Fasciola hepatica* in Great Britain

The work presented in this chapter has drawn upon material from within N. J. BEESLEY, K. CWIKLINSKI, D. J. L. WILLIAMS and J. HODGKINSON, (2015), "Fasciola hepatica from naturally infected sheep and cattle in Great Britain are diploid", (adapted), pp 1196-1201, Parasitology, (2015) © Cambridge University Press 2015, and is reproduced with permission.

2.1 INTRODUCTION

Knowledge of parasite ploidy is important to enhance our understanding of parasite biology, and is essential to increase our knowledge of parasite genetics, reproduction, and the transfer of genes from one parasite population to another. Knowledge of ploidy is also vital to our understanding of genetic diversity of *Fasciola hepatica* populations.

Diploid (2n = 2x = 20) and triploid (2n = 3x = 30) *F. hepatica* have been reported in the UK, and in Asia diploid, triploid and mixoploid (2x/3x) *Fasciola* spp. exist (Sanderson, 1953; Rhee *et al.*, 1987; Fletcher *et al.*, 2004), but there is little information to indicate how common triploidy is, particularly in UK fluke. To analyse ploidy in *F. hepatica*, the rapidly dividing sperm cells can be stained and used to visualise chromosomes. Spermatogenesis in *F. hepatica* follows a series of three mitotic divisions; from one primary spermatogonia to two secondary spermatogonia to four tertiary spermatogonia to an 8-cell rosette of primary spermatocytes, followed by two meiotic divisions to produce 16 secondary spermatocytes (16-cell), then 32 haploid spermatozoa (32-cell; John, 1953; Gresson, 1965; Stitt and Fairweather, 1990). Different techniques exist to visualise the chromosomes and the most frequently used include: aceto-orcein staining (e.g., Fletcher *et al*, 2004), squashing and staining with alcoholic hydrochloric acid-carmine (Itagaki *et al.*, 2009), modified air-drying with Giemsa staining (Rhee *et al.*, 1987) and spread preparations stained with Giemsa (Reblánová *et al.*, 2011).

The aim of this chapter was to determine the proportion of diploid and triploid *F. hepatica* isolated from naturally infected sheep and cattle in Great Britain. The only species of *Fasciola* present in the UK is *F. hepatica* and, to date, studies on the ploidy of *F. hepatica* in the UK have been limited to small numbers of adult parasites (5 to 15 parasites; Fletcher *et al.*, 2004). Knowledge of ploidy is essential to accurately determine genotypes for population level genetic studies and in order to execute the appropriate analysis of population genetic data generated within Chapters 3 and 4 of this thesis. As a number of techniques have been described to visualise chromosomes in *Fasciola* spp., a method for chromosome visualisation was optimised using adult *F. hepatica* collected from sheep. A sample size calculation was performed so that a statistically representative number of parasites were

analysed to allow accurate determination of the proportion of diploids and triploids in the parasite population as a whole. This constitutes the first statistically valid analysis of the ploidy of *F. hepatica* field isolates from Great Britain.

2.2 MATERIALS AND METHODS

2.2.1 Fasciola hepatica *collection*

Adult *F. hepatica* were recovered from the livers of 66 naturally infected lambs between November 2012 and February 2013, from three different abattoirs located in Wales, North West England and Central England. Based on the catchment areas of these abattoirs, these samples represent field populations of *F. hepatica* from England, Wales and Scotland. Adult *F. hepatica* were recovered from 35 cattle livers between October 2013 and January 2014, from an abattoir located in Wales. Based on ear tag information, kindly provided through Rapid Analysis and Detection of Animal Related Risks (RADAR), Animal and Plant Health Agency (APHA), it was determined that these samples represent field populations of *F. hepatica* from England and Wales.

Sheep livers were transported to the University of Liverpool, where adult parasites were isolated from the bile ducts. Parasites from cattle livers were isolated *in situ* at the abattoir. To purge intestinal contents and eggs, the parasites were incubated, for a minimum of 2 hours at 37^{o}C in 1 to 2 ml of Dulbecco's Modified Eagle's Media (Sigma-Aldrich, Dorset, UK) with 120 μgml^{-1} of gentamicin (Sigma-Aldrich) and 120 μgml^{-1} of amphotericin B (Sigma-Aldrich). After incubation, each individual parasite was washed in Dulbecco's Phosphate Buffered Saline (PBS). A total of 580 *F. hepatica* were recovered from sheep with one to 12 parasites obtained from each liver and 150 *F. hepatica* were recovered from cattle with one to seven parasites from each liver.

2.2.2 Determining ploidy of Fasciola hepatica

Since no one standard method exists for determining ploidy a protocol for chromosome visualisation was optimised with the assistance of Dr. Krystyna Cwiklinski. An additional archive of *F. hepatica* was collected from sheep purely for optimisation purposes. Different methods were evaluated following adaptation from Fletcher *et al.* (2004) and Reblánová *et al.* (2011) and included variations of purging time, the use of colchicine and potassium chloride, +/- fixing of samples, and assessment of an aceto-orcein squash or smear technique (Table 2.1). Briefly, for aceto-orcein squash a section of the distal two-thirds of the parasite (approximately 1

mm^3) was macerated in a drop of 3 % (w/v) orcein (Sigma-Aldrich) in 45 % (w/v) acetic acid on a glass slide, and then squashed under a cover slip using filter paper to soak up the excess stain. For aceto-orcein smear, a cut was made horizontally across part of the distal two thirds of the parasite; the cut edges were smeared onto a glass slide to transfer cells. This was then stained by flooding the slide with the aceto-orcein stain. In all instances the aceto-orcein squash technique was more reliable than the aceto-orcein smear.

Whilst it was preferable to use fresh material and perform an aceto-orcein squash immediately after a 2 hour purge of adult *F. hepatica* (method 2, Table 2.1), the practicalities of using large numbers of parasites from sheep necessitated purging for 2 hours followed by incubation in 0.025 % (w/v) colchicine (Sigma-Aldrich) in PBS, pH 7.4 for 1 hour at room temperature (RT); followed by incubation in 75 mM potassium chloride (VWR, Lutterworth, UK) for 1 hour at RT, and fixing (3:1, ethanol: acetic acid) prior to performing aceto-orcein squash (method 16, Table 2.1). The optimal time period to perform aceto-orcein squash following fixation (method 16, Table 2.1) was within two weeks; when fresh samples were processed (method 2, Table 2.1) this was within four hours of collection. All chromosome preparations were examined within 24 hours of preparation. The 580 *F. hepatica* from sheep (see section 2.2.1) were examined using an optimised method for fixed samples (method 16, Table 2.1) whilst the 150 parasites from cattle were examined immediately after purging, i.e. using fresh material (method 2, Table 2.1).

Chromosome preparations were examined using a Zeiss Axio Imager M2 microscope and Zen 2011 software. The stages of spermatogenesis were identified: rosettes (8-cell stage), 16-cell stage, 32-cell stage, and spermatids and sperm (Figure 2.1). The number of chromosomes was counted in well-spread cells (Figure 2.2), and was usually most apparent during the first meiotic division from rosette to 16-cell stage and during late metaphase when the chromosomes were clumped, rather than early metaphase when the chromosomes were elongated. Triploid *F. hepatica* is aspermic or has very few, abnormally developed sperm and does not undergo the same stages of spermatogenesis as diploid parasites (Terasaki *et al.*, 2000; Fletcher *et al.*, 2004; Hanna *et al.*, 2008; Itagaki *et al.*, 2009). Following the criteria of Sanderson, 1953; Fletcher *et al.*, 2004; Hanna *et al.*, 2008; Itagaki *et al.*, 2009, here the presence of sperm was used as a proxy to determine diploidy. A parasite was deemed to be diploid if either 10 bivalent chromosomes or sperm were observed.

Table 2.1: Description of the methods evaluated for optimisation of chromosomal preparation to determine the ploidy of *Fasciola hepatica*

	Method	No. of *F. hepatica* processed
1	Purging[1] 2 h and aceto-orcein smear[2]	8
2	Purging 2 h and aceto-orcein squash[3]	8
3	Purging 2 h, incubation in colchicine[4] at RT 1 h and aceto-orcein smear	8
4	Purging 2 h, incubation in colchicine at RT 1 h and aceto-orcein squash	8
5	Purging 2 h, incubation in colchicine at RT 1 h, fix[5] and aceto-orcein squash	13
6	Purging 2 h, chopping[6], incubation in colchicine at RT 1 h and aceto-orcein squash	8
7	Purging 2 h, chopping, incubation in colchicine at RT 1 h, fix and aceto-orcein squash	12
8	Purging overnight and aceto-orcein smear	4
9	Purging overnight and aceto-orcein squash	4
10	Purging overnight, incubation in colchicine at RT 1 h and aceto-orcein smear	4
11	Purging overnight, incubation in colchicine at RT 1 h and aceto-orcein squash	4
12	Purging overnight, incubation in colchicine at RT 1 h, fix and aceto-orcein squash	5
13	Purging overnight, chopping, incubation in colchicine at RT 1 h and aceto-orcein squash	4
14	Purging overnight, chopping, incubation in colchicine at RT 1 h, fix and aceto-orcein squash	7
15	Purging 2 h, incubation in colchicine at RT 1 h, incubation in potassium chloride[7] at RT 1 h and aceto-orcein squash	10
16	Purging 2 h, incubation in colchicine at RT 1 h, incubation in potassium chloride at RT 1 h, fix and aceto-orcein squash	13
17	Purging 2 h, chopping, incubation in colchicine at RT 1 h, incubation in potassium chloride at RT 1 h and aceto-orcein squash	13
18	Purging 2 h, chopping, incubation in colchicine at RT 1 h, incubation in potassium chloride at RT 1 h, fix and aceto-orcein squash	9

[1.] Incubation at 37°C to allow intestinal contents and eggs to be expelled from the parasite; [2.] 3 % (w/v) orcein in 45 % (w/v) acetic acid was used to macerate parasite tissue and was then squashed under a cover slip; [3.] Parasite cells were smeared onto a slide from a cut edge and stained with 3 % (w/v) orcein in 45 % (w/v) acetic acid; [4.] 0.025 % (w/v) in PBS, pH 7.4; [5.] 3:1, ethanol: acetic acid and stored at 4°C; [6.] The parasite was chopped into smaller sections to allow deeper penetration of the chemicals; [7.] 75 mM; h = hour(s). In the optimisation experiments ploidy could not be determined in 26 (18.3 %) samples. Ploidy was determined in the remainder either by visualisation of chromosomes and sperm (31 samples; 21.8 %); or sperm alone (85 samples; 59.6 %).

2.2.3 Statistical programs

The StatCalc function of Epi Info 7 (http://wwwn.cdc.gov/epiinfo/7/) was used to calculate a statistically valid sample size. Calculations were initially based on an expected proportion of 50 % triploid individuals (to give the highest possible sample size required). A sample size of 384 individuals gave 95 % confidence level with 5 % confidence limits (precision).

2.2.4 Ethical approval

Ethical approval was received from the University of Liverpool's Veterinary Research Ethics Committee (VREC106 and VREC145).

2.3 RESULTS

Ploidy was determined for a total of 565 and 150 *F. hepatica* adult parasites isolated from naturally infected sheep and cattle, respectively. All 715 parasites were diploid. The proportion of triploids identified was 0 % (95 % CI 0, 0.49 %; Hanley and Lippman-Hand, 1983). No difference between the ploidy of parasites isolated from sheep and cattle was found.

Ten bivalent chromosomes (Figure 2.2) and sperm were observed in 335 (46.9 %) of all *F. hepatica* samples; sperm alone were observed in 380 (53.1 %) of the samples. The different stages of spermatogenesis: rosettes (8-cell stage), 16-cell stage, 32-cell stage, and spermatids and / or sperm were observed in 93.3 %, 34.7 %, 72.9 % and 100 % of samples, respectively, as summarised in Table 2.2 (see also Figure 2.1). All stages of spermatogenesis were observed in 144 *F. hepatica* from sheep and 53 *F. hepatica* from cattle (27.6 % of all parasites). Sperm cells were commonly associated with the rosette and 32-cell stages; fewer 16-cell stages were observed. If the 16-cell stages were excluded, all other stages of spermatogenesis (rosette, 32-cell and sperm) were seen in 360 *F. hepatica* from sheep and 146 from cattle (70.8 % of all parasites).

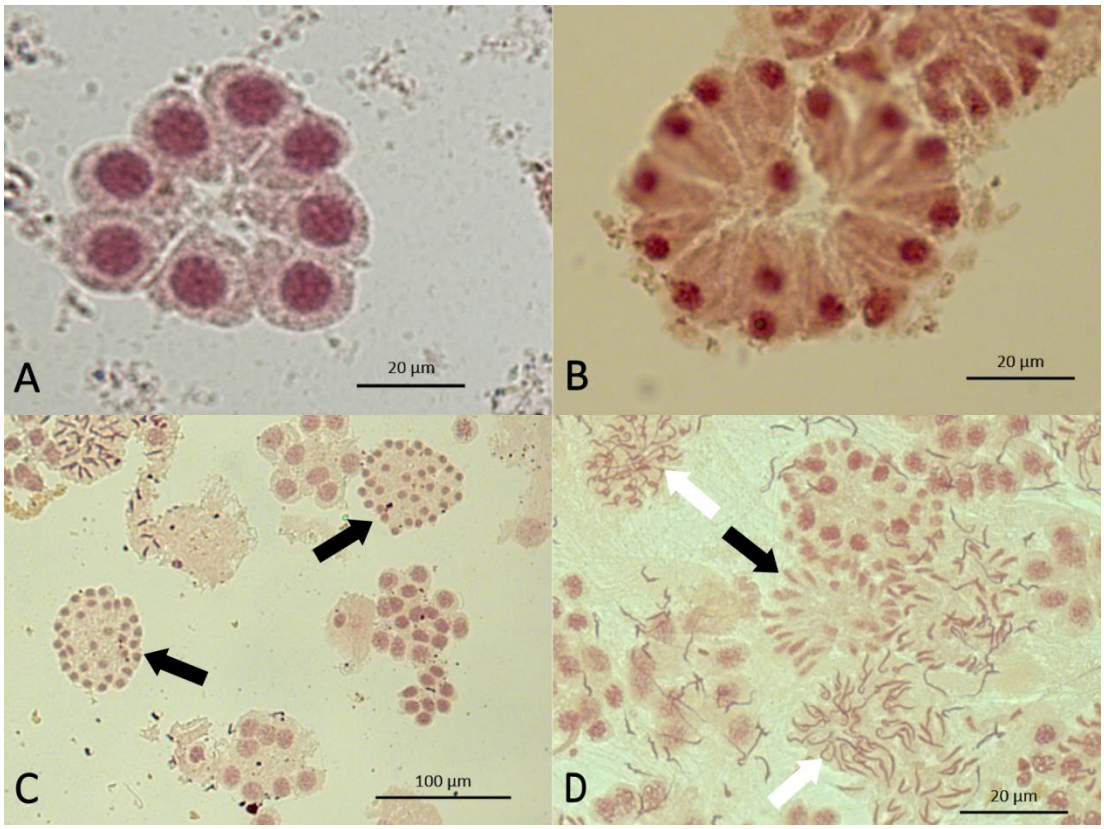

Figure 2.1: The different stages of spermatogenesis in *Fasciola hepatica*; **A**: the 8-cell or rosette stage; **B**: the 16-cell stage; **C**: the 32-cell stage (black arrows); **D**: the nuclei from the 32-cell stage elongate to spermatids (black arrow) and then sperm (white arrows)

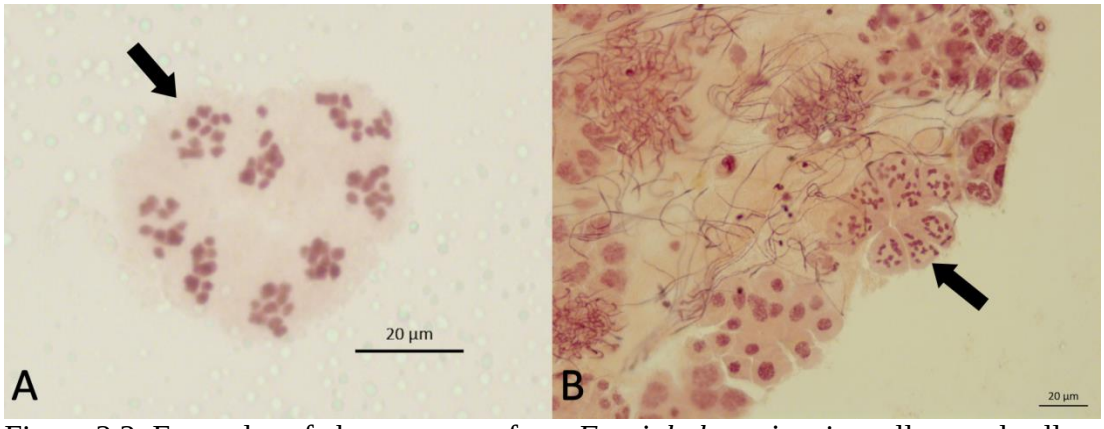

Figure 2.2: Examples of chromosomes from *Fasciola hepatica*; in well-spread cells (arrows) chromosomes can be counted; **A**: *F. hepatica* from a sheep liver; **B**: *F. hepatica* from a cow liver

Table 2.2: Ploidy of wild British *Fasciola hepatica* from sheep and cattle: a breakdown of the spermatic stages observed and details of parasite burden on a species basis

Species	No. of Animals	Median adult *F. hepatica* per liver (range)	No. of *F. hepatica* analysed (range per liver)	No. of parasites in which each stage of spermatogenesis was observed (%)				No. of parasites with 10 bivalent chromosomes (%)	No. of diploid *F. hepatica* (%)
				8-cell	16-cell	32-cell	sperm		
Sheep	66	10*	565	517	194	375	565	246	565
		(1 – >100)	(1 – 12)	(91.5)	(34.3)	(66.4)	(100)	(43.5)	(100)
Cattle	35	15	150	150	54	146	150	89	150
		(1 – 133)	(1 – 7)	(100)	(36)	(97.3)	(100)	(59.3)	(100)
Total	101	11.5*	715	667	248	521	715	335	715
				(93.3)	(34.7)	(72.9)	(100)	(46.9)	(100)

*Total enumeration of parasites was not determined for 19 of the sheep livers, therefore these figures are based on 47 of the 66 sheep

2.4 DISCUSSION

2.4.1 Fasciola hepatica *in Great Britain are diploid*

The *F. hepatica* samples analysed in this study were isolated from naturally infected sheep and cattle from England, Wales and Scotland. Ploidy was determined for 715 parasites, all of which were diploid, as shown by detection of 10 bivalent chromosomes and sperm, or sperm alone. The number of parasites sampled is almost twice the 384 individuals required to give 95 % confidence level with 5 % confidence limits of the proportions of diploid and triploid parasites in Britain, and can therefore be considered representative of field populations in Great Britain. Furthermore, these results are consistent with other studies of ploidy in wild populations of *F. hepatica*. In Northern Ireland, a smaller scale study identified diploid parasites (n = 15) isolated from naturally infected cattle (Fletcher *et al.*, 2004) and similarly a study in Slovakia which analysed 10 parasites from a cattle liver found exclusively diploid organisms (Reblánová *et al.*, 2011). Overall the results presented here suggest that triploidy in *F. hepatica* populations, at least in Great Britain, is rare to non-existent. The presence of sperm in all of the mature parasites studied here is in contrast to *Fasciola* spp. in Asian populations which have frequently been described as aspermic or, triploid and aspermic (Itagaki *et al.*, 1998; 2009; Terasaki *et al.*, 1998; 2000), and infers that sexual reproduction, rather than parthenogenesis, is the most frequent means of reproduction in *F. hepatica* populations from Great Britain.

Although diploid *Fasciola* spp. with no or abnormal sperm have been described (Terasaki *et al*; 1998; 2000, Hanna *et al.*, 2008), these were in parasites from Asian countries where *F. hepatica* and *F. gigantica* co-exist, or from the Sligo laboratory isolate. In our study, reported here, a parasite was described as diploid based on observation of 10 bivalent chromosomes and sperm, or the presence of sperm alone since triploid *F. hepatica* are aspermic, or have small numbers of morphologically abnormal sperm (Terasaki *et al.*, 2000; Fletcher *et al.*, 2004; Hanna *et al.*, 2008; Itagaki *et al.*, 2009). Abnormal spermatogenesis is also a feature commonly applied to triploid *Fasciola* spp. (Itagaki *et al.*, 1998; 2009) so here we observed not just sperm but all stages of spermatogenesis. Determining a parasite diploid based on the presence of sperm alone was used in only 43 (6.0 %) samples;

sperm plus one, two or all other stages of spermatogenesis (8-cell, 16-cell, 32-cell) were seen in 119 (16.6 %), 356 (49.8 %) and 197 (27.6 %) parasites, respectively. Similar to the findings of others (Stitt and Fairweather, 1990) the 16-cell stage was the least observed stage, seen in only 34.7 % of parasites, and supports the idea that this stage is "short-lived" (Stitt and Fairweather, 1990).

2.4.2 The likelihood of triploid Fasciola hepatica *occurring in Great Britain*

Different isoenzyme electrophoretic patterns provide evidence that triploidy in Japanese *Fasciola* spp. has arisen independently on more than one occasion (Agatsuma *et al.*, 1994). The majority of sequencing evidence supports the hypothesis that triploids are the result of hybridisation between *F. hepatica* and *F. gigantica* in areas where their distribution overlaps (Itagaki *et al.*, 1998; 2005a; 2005b; 2009; Agatsuma *et al.*, 2000; Peng *et al.*, 2009; Mohanta *et al.*, 2014). In Egypt, *F. hepatica* and *F. gigantica* have been found in the same definitive host (Amer *et al.*, 2011); this presents the opportunity for hybridisation between the two species, and confirmation of the ability of *F. hepatica* and *F. gigantica* to cross-fertilise using experimental systems supports this hypothesis (Itagaki *et al.*, 2011). Triploids are assumed to be maintained by parthenogenesis (Terasaki *et al.*, 1998; 2000; Itagaki *et al.*, 2005a; Hanna *et al.*, 2008); in areas where co-infection occurs, the ability of hybrid triploids to undergo parthenogenesis allows establishment of clonal field populations (Walker *et al.*, 2012), which has implications for genetic variation.

There is one report of triploidy in *F. hepatica* in the UK, in the Cullompton laboratory isolate. This isolate was derived in 1998 from multiple eggs obtained from the bile ducts of several sheep, which were used to infect snails, and then passaged twice through sheep and once through rats. The analysis of ploidy on this isolate was not performed until between 2001 and 2003 (Fletcher *et al.*, 2004). Since UK populations of *Fasciola* spp. are restricted to *F. hepatica* it is unlikely that triploids form by hybridisation. Our results suggest that triploidy in the Cullompton isolate is an artefact of isolation and laboratory passage, rather than a reflection of events taking place in the field. It is reported the Cullompton isolate was passaged several times following isolation; potentially creating a bottleneck. Passage was also through rats; a non-natural host of *F. hepatica* with smaller livers able to support far fewer individual parasites compared to sheep and cattle, which may have increased

stress on the parasites. Whilst we do not know the effect long term laboratory passage has on parasites, these stresses may have led to the induction of triploidy in the Cullompton isolate. It is possible to induce polyploidy in helminths: heat shock induced polyploidy in *Caenorhabditis elegans* producing tetraploids from diploids, probably via a triploid (Madl and Herman, 1979).

Other stressors have also been shown to alter ploidy in vertebrates. For example, in bovine embryos the chemical cytochalasin B induced changes in ploidy (Bai *et al.*, 2011), and in leopard frogs, *Rana pipiens*, hydrostatic pressure has a similar affect (Dasgupta, 1962). In plant species, changes to environmental temperatures (Pécrix *et al.*, 2011), wounding, and water or nutrient stress (Ramsey and Schemske, 1998) have also been shown to alter ploidy. If one of these stressors produced a diploid female gamete (unreduced female gametes are more common than unreduced male gametes; Otto and Whitton, 2000), a triploid would be produced following fertilisation with a haploid sperm. In order to maintain triploidy over successive generations, the initial creation of an accidental triploid would have to be derived from a parthenogenic diploid (Terasaki *et al.*, 2000).

Knowledge of the frequency of parthenogenesis in *Fasciola* spp. is limited and also complicated by the fact that *Fasciola* spp. are hermaphrodites that can self-fertilise. To our knowledge it is only assumed that the Cullompton isolate undergoes parthenogenesis, and this has not been proven by analysing physical (i.e. presence [or not] of sperm in the seminal vesicle and uterus) and genetic characteristics (i.e. sequencing or genotyping) in tandem. The Cullompton isolate was reported to have 30 univalent chromosomes (Fletcher *et al.*, 2004), which is consistent with the observations of abnormal spermatogenesis and potential for parthenogenesis in Asian *Fasciola* spp. (Terasaki *et al.*, 1998; 2000; Itagaki *et al.*, 2005a). Whilst the Cullompton isolate was originally generated from multiple eggs from multiple animals suggesting at least some sort of genetic diversity, if at some point overall parasite numbers reduced to one parasite following passage through rats this could explain how triploidy was created and then maintained across the entire isolate. Interestingly some specimens of the Sligo isolate have been shown to be aspermic diploids (Hanna *et al.*, 2008), and this may represent the first step towards parthenogenesis. However since the experiments undertaken on the Sligo and Cullompton isolates were several years after isolation we do not know the events leading up to these observations.

Fletcher *et al.*, (2004) also suggested that triploidy could be produced by the fertilisation of a haploid egg with two haploid sperm. However, in another study of *F. hepatica*, whilst more than one sperm became enclosed within the capsule of the egg, more than one sperm was never observed in the ooplasm (Sanderson, 1959). A further possibility is the introduction of triploids, from an endemic area into the UK. Although there is evidence that triploid *Fasciola* spp. can stabilise within a population once introduced (Itagaki *et al.*, 2005a; 2009; Mohanta *et al.*, 2014), the UK does not routinely import from such endemic areas.

Despite being triploid and aspermic, the infectivity of the Cullompton isolate has not been affected, since recent experiments have demonstrated that the isolate will successfully infect sheep and cattle (Hanna *et al.*, 2008). It is suggested that since triploids in essence have more genetic material, the rate of mutation would be higher and this would be an advantage for the development and spread of resistance (Fletcher *et al.*, 2004). Yet the Cullompton strain is susceptible to triclabendazole (McCoy *et al.*, 2005; Halferty *et al.*, 2008; McConville *et al.*, 2009), and since much of this work has been done after the elucidation of the ploidy of the Cullompton strain, it can be extrapolated that triploidy has not yet conferred an advantage in this respect. Whilst new mutations are more likely due to the increase in genetic material there is also a dilution effect, so method of reproduction, population size and dominance also influence any advantage polyploidy may confer (Otto and Whitton, 2000). Laboratory isolates are often not representative of what is happening in the field; and knowledge of the provenance of isolates, their maintenance within the laboratory in terms of passage and continued assessment of their infectivity, pathogenicity, and resistance status is an essential part of studies on *F. hepatica*, but is frequently overlooked (Hodgkinson *et al.*, 2013).

2.4.3 Conclusion

Since the study reported here has shown that a statistically meaningful number of *F. hepatica*, from naturally infected sheep and cattle in Great Britain, were diploid, triploidy, if it exists, is rare. These results suggest that triploidy is most likely to develop from hybridisation between *F. hepatica* and *F. gigantica*. If this is the case, it means that the results of this study may be extrapolated to other countries where, like the UK, *F. hepatica* exists in isolation from its sister species *F. gigantica*, and that triploidy is most common in areas where co-infection of *F. hepatica* and *F.*

gigantica occurs. This, taken with the observation that sexual reproduction was more frequent than parthenogenesis (since all the parasites studied here contained sperm), will enable further genetic and molecular studies of *F. hepatica* populations. Knowledge that *F. hepatica* populations in the UK are diploid is essential to support assumptions about inheritance patterns and for the calculation of observed and expected allele frequencies that are fundamental to population genetic studies (Dufresne *et al*., 2014).

CHAPTER 3

A population genetic study of *Fasciola hepatica* from naturally infected sheep and cattle in Great Britain

3.1 INTRODUCTION

Population genetic analyses help us to understand the origin, evolution, and spread of resistance genes in populations; and are thus a vital component of anthelmintic resistance studies (Gilleard and Beech, 2007). A number of studies have analysed the population genetic structure of *Fasciola hepatica* in Europe, Asia and Australia, and report that *F. hepatica* populations are panmictic with high genetic variability and high gene flow (Morozova *et al.*, 2004; Semyenova *et al.*, 2006; Walker *et al.*, 2007; 2011; Vázquez-Prieto *et al.*, 2011; Vilas *et al.*, 2012; Elliott *et al.*, 2014; see Chapter 1). The largest of these studies by Vilas *et al.*, (2012) analysed 587 adult *F. hepatica* and showed different patterns of population genetic structure between sheep and cattle. However, since these parasites were taken from a limited number of animals from the same farm (sheep) or different farms (cattle), it is difficult to compare the population genetic structure of *F. hepatica* between definitive host species. These studies are also limited by the genetic markers they use: allozymes may not detect the true extent of genetic differentiation and mitochondrial DNA markers show only maternal inheritance and can be influenced by selection (Beveridge, 1998; Galtier *et al.*, 2009).

A number of aspects of *F. hepatica* biology have the potential to affect the spread of genes, including those that may be responsible for anthelmintic resistance (Hodgkinson *et al.*, 2013). It is known that clonal expansion occurs within the snail intermediate host (Thomas, 1883), and Vilas *et al.*, (2012) found parasites with the same MLG within, and shared between, definitive hosts. As a hermaphrodite, *F. hepatica* can self- and cross-fertilise. Self-fertilisation is a form of inbreeding which has the potential to influence allele frequency in a population. If anthelmintic resistance is a recessive trait, a high level of self-fertilisation would mean there was the potential for resistant alleles to spread more rapidly than if cross-fertilisation predominates. Animal husbandry and management factors influence the movement of the definitive host and, therefore, *F. hepatica* parasites. The age and production process of an animal influences the extent to which it has been exposed to *F. hepatica* on pasture and to what extent it may have been treated with anthelmintics. Together with the fact that *F. hepatica* can be long-lived this means adult parasites may be present for many years in untreated animals.

The population genetic structure of *F. hepatica* in Great Britain is not known, yet as discussed in Chapter 1, the problem of triclabendazole resistance in *F. hepatica* is increasing throughout the UK. Knowledge of population genetic structure will allow us to better understand the movement of drug resistance genes, identify management factors influencing their migration, and allow us to mitigate against their spread.

The first aim of this chapter was to determine the level of genetic diversity in the British *F.* hepatica population. The second aim of this chapter was to determine whether parasites of the same MLG are found within the definitive host, namely sheep and cattle in Britain. The third aim was to evaluate the population structure of the British *F. hepatica* population to define whether there is evidence for geographical structuring, and if there are genetic differences between parasites infecting sheep and cattle. Adult parasites were collected from sheep and cattle at point of slaughter and were genotyped with our validated panel of microsatellites (Cwiklinski *et al.*, 2015a), which was optimised as a multiplex PCR approach to allow a high throughput of samples.

3.2 MATERIALS AND METHODS

3.2.1 Fasciola hepatica *collection*

Adult *F. hepatica* were recovered from the livers of 44 naturally infected sheep between November 2012 and April 2013, from two different abattoirs located in Wales and Central England. Adult *F. hepatica* were recovered from 31 cattle livers between October 2013 and January 2014, from an abattoir located in Wales. Parasites were isolated as described in Chapter 2.2.1. After purging of intestinal contents and eggs, parasites were frozen and stored at -80^{o}C for DNA extraction at a later date. A total of 950 parasites were recovered from sheep and 629 from cattle; further details are shown in Table 3.1.

Table 3.1: Details of *Fasciola hepatica* collected from sheep and cattle

Species	**No. of animals**	**Demographic information**	**Median burden (range)**	**No. of parasites genotyped (median; range per liver)**
Sheep[1]	8	Scotland[2]		288[3]
Sheep[1]	5	Wales[2]	69	180[3]
Sheep[1]	1	England[2]	(36 – >200)	36[3]
Sheep[1]	6	England or Wales[2]		216[3]
Sheep[1]	24	5 farms local to the abattoir in Wales or Central England	9.5[4] (3 – 100)	230 (10.5; 2 – 18)
Cattle	1	England	Mixture of males and females, beef and dairy breeds, median age 8.5 years (range 2.0 to 16.6)[5]	13
Cattle	30	21 farms in Wales	19 (1 – >230)	616 (18; 1 – 36)

[1.] sheep samples came from lambs (approximately 6 to 12 months old) that had grazed for only one season (typically grazed for 3 to 9 months, but no longer than one year; exposed to *F. hepatica* metacercariae summer and autumn 2012); [2.] these lambs were purchased by the abattoir in Central England from markets with the owner providing information on the country of origin; [3.] 36 parasites were sampled from each animal; [4.] total enumeration was not performed for six animals; [5.] this information was provided through RADAR, APHA using ear tag numbers

3.2.2 DNA extraction

The parasites were thawed and a section (approximately 25 mg) from the proximal end of the parasite, to ensure that no reproductive cells were isolated, was used for DNA extraction. The tissue was divided into small pieces using a scalpel to ensure efficient lysis. DNA extraction was performed using a DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. The lysis step took approximately 1 hour to complete. All reagents were included in the kit except for molecular grade ethanol (Sigma-Aldrich).

3.2.3 DNA dilution: precipitation and quantification

All samples were precipitated by adding 0.1 volumes of 3M sodium acetate, pH 5.2[1] and 0.7 volumes of isopropanol (Sigma-Aldrich). Samples were spun at 6500 g for 30 minutes at 4^{o}C and the supernatant was discarded. The sample was washed by spinning at 6500 g for 20 minutes at 4^{o}C with 1 ml of ice cold 70 % molecular grade ethanol. The ethanol was removed and the DNA pellet visualised. The pellet was left to air dry for approximately 10 minutes depending on its size, and then re-suspended in TE[2] buffer. Re-suspension was aided by leaving for 1 to 2 hours at 55^{o}C in a shaking incubator at 30 rpm.

DNA was quantified using the Quant-it Pico Green dsDNA Assay kit (Life Technologies, Warrington, UK). A standard curve was created with a DNA standard and TE at 0, 1, 10, 100 and 1000 ngml^{-1}. The fluorescence of the samples was compared to that of the standard curve using a Tecan Infinite F200 and Magellan 7. Due to the number of samples, the size of the DNA pellet produced was visualised following precipitation. Quantification was performed for a proportion of samples with pellets of different sizes. Based on this quantification DNA was diluted using TE to a final concentration of 10 ngµl^{-1}. For those samples that were not quantified a dilution was made based on the size of the pellet in comparison to those that had been quantified.

[1] 3M Sodium Acetate pH 5.2: 408.3g of sodium acetate·3H_2O in 800 ml of water, pH adjusted using glacial acetic acid, autoclaved

[2] 10X TE pH 8.0: 100 mM Tris-Cl (pH 8.0) , 10 mM EDTA (pH 8.0), autoclaved
1 M Tris-Cl pH 8.0: 121.1 g of Tris base in 800 ml of water plus 42 ml of concentrated HCl
0.5 M EDTA (pH 8.0) 186.1g of disodium EDTA·2H_2O to 800 ml of water, adjust pH to 8.0 with NaOH. autoclaved

3.2.4 Producing a multilocus genotype (MLG)

3.2.4.1 Optimisation of a multiplex PCR

A panel of fifteen microsatellites had previously been validated on 46 adult *F. hepatica* (Cwiklinski *et al.*, 2015a). A semi-nested PCR protocol based on Schuelke's method was used to amplify these microsatellites whereby the fluorescent forward primer is added in the second round of PCR via an M13 tag (Schuelke, 2000). To improve the efficiency of genotyping this method was optimised for a multiplex PCR approach.

The first step to produce a multiplex PCR was to ensure that direct labelling of the forward primer with fluorescence did not affect the PCR (primer sequences are shown in Table 3.2). Each microsatellite was compared in singleplex using three samples of adult *F. hepatica* DNA previously used for validation by Cwiklinski *et al.*, (2015a). The PCR reaction and PCR conditions were as described for primary amplification by Cwiklinski *et al.*, (2015a) except that the number of cycles was increased to 30. The primer sets were then combined based on those with similar annealing temperatures and tested in a multiplex reaction at different temperatures (Table 3.2). Each multiplex set was compared using three samples of adult *F. hepatica* DNA previously used for the original validation. The temperature producing optimal results was chosen for future reactions. The Type-it Microsatellite PCR kit (Qiagen) was used according to the manufacturer's instructions. The PCR reaction was 25 µl: 12.5 µl Type-it Microsatellite Buffer (final concentration 1X), 2.5 µl of the primer mix (final concentration of each primer 0.2 µM), 10 ng of template DNA and made up to 25 µl with RNAse free water. The PCR reaction conditions were an initial denaturation stage of 95^{o}C for 5 minutes; 30 cycles of 95^{o}C for 30 seconds, annealing temperature (Table 3.2) for 90 seconds, and 72^{o}C for 30 seconds; and a final extension stage of 60^{o}C for 30 minutes. All PCR products were visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5 % agarose gel run at 150 V. After processing 360 parasites from sheep it was found that amplifying locus Fh_1 in the multiplex produced a number of artefacts that made the traces from the capillary sequencer difficult to read. Therefore for a further 360 sheep samples this locus was performed in singleplex. Initial analysis showed the presence of null alleles at locus Fh_1, therefore a genotype for locus Fh_1 was not produced for the remaining sheep samples, nor for any of the cattle samples.

3.2.4.2 Capillary electrophoresis to determine fragment size

Any PCR products that produced a positive result when visualised by gel electrophoresis were diluted 25-fold in HPLC water (Sigma-Aldrich). The diluted PCR products were sequenced using an ABI PRISM 3100 Genetic Analyser capillary electrophoresis system (Life Technologies), the reaction mixture contained 1 µl of the diluted PCR product multiplexed in Hi-Di Formamide (8.8 µl; Life Technologies) with GeneScan LIZ500 as the size standard (0.2 µl; Life Technologies).

Fragment sizes were determined by analysing the data produced from the capillary sequencer using Peak Scanner v2.0 software (Life Technologies), and a MLG was produced for each individual parasite. Periodically throughout the data collection, all results were reviewed to ensure that they were being interpreted in a consistent manner. Locus Fh_9 did not produce consistent traces, and was therefore excluded from the statistical analyses due to technical error.

Table 3.2: Details of the microsatellite panel including primer sequences and annealing temperatures for singleplex and multiplex reactions

Locus	Type of Repeat	PCR fragment (allele) size range / bp	Forward Primer Sequence – fluorescently labelled (Applied Biosystems)	Reverse Primer Sequence (Eurofins MWG Operon)	Dye	ST_a / ^oC	Multiplex Group	MT_a / ^oC (MT_a / ^oC trialled)
Fh_1	Tetra	185 – 233	5’-CCCATGGTGTTGCACAGAT-3’	5’-CTTCACCAAAAGCCGCTAAC-3’	VIC	55	A*	55
Fh_6	Tri	159 – 252	5’-ACGTCCGTCCGTTAAGTGAG-3’	5’-TTTGAGGTCGACATCCTTCA-3’	6-FAM	55	A	55 (55)
Fh_13	Tri	183 – 222	5’-GAAACTGTCCCGAAAACGAG-3’	5’-GCGTGCAACATAGGTGAAAA-3’	PET	55		
Fh_15	Tetra	198 – 274	5’-AATGCGGAAAAGAGCGATTA-3’	5’-GAAATTGGGAGCAACTGCAT-3’	NED	55		
Fh_2	Tetra	165 – 353	5’-TGAGAAACTGATTCACCGACTG-3’	5’-GAGCTTGTGCTCTCGGAACTA- 3’	VIC	57	B	57 (57 and 58)
Fh_3	Tetra	176 – 200	5’-CACGGCAACTGATGAATGAA-3’	5’-TCCTCGTTTTTGGACCTCAG-3’	PET	58		
Fh_5	Tri	141 – 327	5’-CATCACCACTGTCTTCGATCA-3’	5’-CGAAGCATTGATAAGATTTCC-3’	6-FAM	57		
Fh_8	Tri	152 – 197	5’-CTCCTGAGGATGATCGGAAA-3’	5’-CTACCGGATCGTTTTGACCA-3’	NED	57		
Fh_9	Tri	191 – 194	5’-AACCCGTATCACCACCAAAC-3’	5’-CTCCCAATCCTGCCATACAT-3’	PET	58	C	57 (57 and 58)
Fh_10	Tri	193 – 241	5’-TTTAGTCGCGGAGCTACCAT-3’	5’-CCACTTTCGTCATGCACATT-3’	NED	57		
Fh_11	Tri	184 – 232	5’-TAAACCGTTGCTTCACGTTG-3’	5’-CAAAGTGTTTGGCGAGCTG-3’	6-FAM	57		
Fh_14	Tri	183 – 240	5’-ACAGGGCTTTGAAGCATGAC-3’	5’-GGGATAGAGCCGTACTGGAA-3’	VIC	57		
Fh_4	Tri	159 – 210	5’-TCACGAAATTGGAGACGACA-3’	5’-TGCATGCAAGAATTGTACCC-3’	PET	59	D	59 (57, 58, 59, 60)
Fh_7	Tri	159 – 207	5’-TGCACTCTAGCATGGTTTGG-3’	5’-AAGTCTTCAGTGCCCCTTCC-3’	NED	57		
Fh_12	Tri	200 – 245	5’-CCACGAGAAGTGGAATTCGT-3’	5’-GTAGGTCCACTCCCTGTCCA-3’	VIC	60		

ST_a: singleplex annealing temperature; MT_a: multiplex annealing temperature; * Fh_1 produced a number of artefacts when included in the multiplex so was usually run in singleplex

3.2.5 Population genetic analyses

3.2.5.1 Calculation of allele and genotype frequencies

For all loci, allele frequencies were determined using CERVUS 3.0.7 (Kalinowski *et al.*, 2007; available from www.fieldgenetics.com) whilst genotype frequencies were determined using GENEPOP 4.2.1 (Rousset, 2008; available from http://kimura.univ-montp2.fr/~rousset/Genepop.htm).

3.2.5.2 Calculating the frequency of null alleles at each locus

Null allele frequency was determined using CERVUS 3.0.7 (Kalinowski *et al.*, 2007). CERVUS 3.0.7 uses an iterative likelihood approach to determine the frequency of null alleles (Summers and Amos, 1997; Marshall *et al.*, 1998; Dakin and Avise, 2004). Loci Fh_1, Fh_3, Fh_4, Fh_7, Fh_8 and Fh_14 were identified as having greater than 5 % frequency of null alleles (Table 3.3). If a null allele is present at a locus this can make a heterozygote locus appear as a homozygote locus. Therefore these loci were excluded from the remaining analyses.

3.2.5.3 Calculation of heterozygosity

Heterozygosity was determined for each individual parasite based on the proportion of loci that were heterozygous. Average heterozygosities were determined for each locus using Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). Unbiased heterozygosity was calculated using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). Mann-Whitney tests were performed using Minitab 17.

3.2.5.4 Identification of parasites with repeated MLGs

GenClone 2.0 (Arnaud-Haond and Belkhir, 2007) was used to identify any repeated MLGs and calculate corresponding P_{sex} values. A repeated MLG is defined as two, or more, parasites sharing an identical MLG. The P_{sex} is the probability that a MLG is derived from a distinct reproductive event rather than being from the same clonal lineage. P_{sex} may be underestimated in instances of selfing. Therefore F_{IS} values, calculated using a round-robin approach to avoid overestimation of any rare allele frequencies (Parks and Werth, 1993), were used to adjust the P_{sex}. Animals from the same farms, or that shared repeated MLGs, were grouped when calculating P_{sex} values.

To determine whether repeated MLGs tended to co-occur in the same host (Gregorius, 2005; Criscione *et al.*, 2011; Vilas *et al.*, 2012) a contingency table was created as described by Vilas *et al.*, (2012), and Fisher's exact test with a Monte Carlo simulation (5000 replicates) was performed using R 3.0.1 (R Core Team, 2013). All parasites were analysed together, along with parasites from sheep and cattle separately. Animals known to come from the same farm were grouped and also analysed with *p*-values corrected using a Bonferroni correction.

The presence of repeated MLGs might make alleles appear more common and affect population genetic structure analyses. Therefore for the remaining analyses comparisons were made between either including repeated MLGs or reducing repeated MLGs to one instance.

3.2.5.5 Determining deviations from Hardy-Weinberg equilibrium

Deviations from Hardy-Weinberg equilibrium were calculated using GENEPOP 4.2.1 (Rousset, 2008). As global tests using Fisher's exact test can inflate the type I error even with large samples (Wigginton *et al.*, 2005), the method used was a two tailed exact test with Markov Chain algorithm (10,000 dememorization, 250 batches, 5000 iterations). Due to the number of tests *p*-values were corrected and compared using (i) Bonferroni correction and (ii) false discovery rate correction (Benjamini and Hochberg, 1995) the latter performed using R 3.0.1 (R Core Team, 2013).

To determine the extent of any significant deviation from Hardy-Weinberg equilibrium, F_{IS} values (Weir and Cockerham, 1984) and differences in the absolute numbers of expected and observed homozygotes / heterozygotes were calculated using GENEPOP 4.2.1 (Rousset, 2008)

3.2.5.6 Determining whether any pairs of loci showed linkage disequilibrium

All pairs of loci were assessed for linkage disequilibrium using GENEPOP 4.2.1 (Rousset, 2008). The null hypothesis states that genotypes at one locus are independent from genotypes at the other locus. All parasites were analysed together, along with parasites from sheep and cattle separately, and, due to the number of tests, *p*-values were corrected and compared using (i) Bonferroni correction and (ii) false discovery rate correction (Benjamini and Hochberg, 1995) the latter performed using R 3.0.1 (R Core Team, 2013).

To demonstrate the extent of linkage disequilibrium for any pairs of loci with significant *p*-values, r^2 values were calculated. To calculate this value knowledge of the gametic phase is needed. Since this is unknown here, the ELB algorithm (Excoffier *et al.*, 2003) was used to imbibe the gametic phase. These calculations were performed using Arlequin 3.5.1.3 (Excoffier and Lischer, 2010).

3.2.5.7 Calculation of genetic diversity

Two measures were used to describe genetic diversity, calculated using GenClone 2.0 (Arnaud-Haond and Belkhir, 2007): (i) genotypic richness (Dorken and Eckert, 2001); (ii) the Pareto distribution (Arnaud-Haond *et al.*, 2007). When calculating genotypic richness animals from the same farms, or that shared repeated MLGs, were grouped. The Pareto distribution was calculated for parasites from sheep and cattle separately and together. R 3.0.1 (R Core Team, 2013) was used to calculate r^2 values and create a linear model to determine if there was any difference between the Pareto distribution for parasites from sheep and cattle. Mann-Whitney tests were performed using Minitab 17.

3.2.5.8 Calculation of F-statistics

F_{IS} and F_{ST} values were calculated using GENEPOP 4.2.1 (Rousset, 2008). F_{IS} was estimated according to Weir and Cockerham (1984) and confidence intervals were calculated using FSTAT 2.9.3 (Goudet, 1995; available from http://www2.unil.ch/popgen/softwares/fstat.htm). F_{ST} was estimated by a weighted analysis of variance (Weir and Cockerham, 1984). Pairwise F_{ST} values were calculated using Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). Principle component analysis (PCA) of these values was performed in R 3.0.1 (R Core Team, 2013), and the package ggplot2 was used to plot results.

3.2.5.9 Calculation of the average number of migrants

Private alleles and private genotypes (ones that are found in a single population only) were identified using GenAlEx 6.5 (Peakall and Smouse, 2006; 2012, available from http://biology-assets.anu.edu.au/GenAlEx/Welcome.html). GENEPOP 4.2.1 (Rousset, 2008) was used to produce a measure for the average number of migrants between populations (N_m) using the private allele method

developed by Slatkin, (1985). For this calculation parasites were grouped according to the definitive host they came from.

3.2.5.10 Testing for isolation by distance

Isolation by distance testing was possible for parasites from cattle only, as farm location was known from ear tag information. Parasites were grouped into populations dependent upon which farm they came from. Isolation by distance was then tested for using GENEPOP 4.2.1 (Rousset, 2008). A Mantel test (5000 permutations) was performed using log transformed geographical distances (as the habitat is multi- not uni-dimensional). Pairwise estimates were related to F_{ST} / (1-F_{ST}), and the minimum geographic distance was set at 0.0001 so that pairwise comparisons between parasites from the same farm were not performed. Ninety-five percent confidence intervals around the regression slope were computed using an ABC bootstrap procedure. Data was plotted in R 3.0.1 (R Core Team, 2013) using the package ggplot2.

3.2.5.11 Inferring population structure

Structure 2.3.4 (Pritchard *et al.*, 2000; available from http://pritchardlab.stanford.edu/structure.html) was used to detect population structure. For the ancestry model, the admixture model (with default settings) was chosen which allows for individuals having mixed ancestry. For the frequency model allele frequencies were correlated among populations and the default settings were used. Burnin length was set at 200,000 and was followed by 100,000 Markov Chain Monte Carlo repeats. K was set at 1 to 47 (the number of farms animals came from) and repeated 20 times. To determine the most appropriate value for K, ΔK was determined using the method proposed by Evanno *et al.*, 2005, and calculated using STRUCTURE HARVESTER (Earl and vonHoldt, 2012; available from http://taylor0.biology.ucla.edu/structureHarvester/). Data was plotted in R 3.0.1 (R Core Team, 2013) using the packages ggplot2 and gridExtra.

A neighbour-joining tree (Saitou and Nei, 1987) using the D_A (Nei *et al.*, 1983) measure of genetic distance between populations was constructed using POPTREE2 (Takezaki *et al.*, 2010; available from http://www.med.kagawa-u.ac.jp/~genomelb/takezaki/poptree2/index.html). Bootstrap tests (Felsenstein, 1985)

were performed 1000 times for the tree. The tree was visualised and graphically edited using FigTree 1.4.2 (available from http://tree.bio.ed.ac.uk/software/figtree/).

3.2.6 Ethical Approval

Ethical approval was received from the University of Liverpool's Veterinary Research Ethics Committee (VREC106 and VREC145).

3.3 RESULTS

A multiplex PCR was successfully optimised to amplify the panel of microsatellites (Table 3.2) and summary statistics are shown for the panel in Table 3.3. Six loci (Fh_1, Fh_3, Fh_4, Fh_7, Fh_8 and Fh_14) showed greater than 5 % frequency of null alleles and were excluded from analyses along with locus Fh_9 (which was excluded due to technical difficulties). Based on the remaining eight loci, a MLG was successfully produced for 99.7 % of parasites: it was not possible to produce a MLG for five parasites.

Table 3.3: Summary statistics for the microsatellite panel based on 1579 parasites

Locus	Frequency of null alleles*	No. of alleles exhibited	No. of genotypes exhibited	H_{obs} / H_{nb}	F_{IS} with repeated MLGs**	F_{IS} without repeated MLGs**
Fh_1	**0.5922**^	9^	17^	ND	ND	ND
Fh_2	0.0112	28	109	0.823 / 0.843	**0.0244**[12]	**0.0299**[12]
Fh_3	**0.1252**	7	17	ND	ND	ND
Fh_4	**0.0753**	16	83	ND	ND	ND
Fh_5	0.0097	39	177	0.852 / 0.867	**0.0184**[2]	0.0199
Fh_6	0.0098	30	178	0.885 / 0.903	**0.0196**[12]	0.0082
Fh_7	**0.1051**	11	37	ND	ND	ND
Fh_8	**0.2255**	16	55	ND	ND	ND
Fh_9	-0.1378	2	3	ND	ND	ND
Fh_10	0.0160	17	75	0.797 / 0.823	**0.0310**[2]	0.0327
Fh_11	0.0237	15	68	0.802 / 0.840	**0.0427**[12]	0.0442
Fh_12	0.0051	15	66	0.733 / 0.740	0.0061	0.0061
Fh_13	-0.0058	12	28	0.633 / 0.628	-0.0084	0.0006
Fh_14	**0.2794**	18	75	ND	ND	ND
Fh_15	0.0064	10	21	0.494 / 0.505	0.0137	0.0198

* calculated using CERVUS 3.0.7 (Kalinowski *et al.*, 2007), results in bold indicate greater than 5 % null allele frequency; H_{obs} = observed heterozygosity; H_{nb} = unbiased heterozygosity; MLGs = multilocus genotypes; ** F_{IS} values are given to indicate deviations from Hardy-Weinberg equilibrium with those results in bold indicating significant *p*-values when using the two-tailed exact test – a Bonferroni and false discovery rate correction were applied [1] = significant when Bonferroni correction applied ($p = 0.00625$) [2] = significant when false discovery rate correction applied; ^ values for locus Fh_1 were determined for 720 of the parasites from sheep only; ND = not determined

As shown in Table 3.3 the majority of significant deviations from Hardy-Weinberg equilibrium were due to the inclusion of repeated MLGs, which would artificially increase the frequency of some alleles. When these repeated MLGs were reduced to one instance, only locus (Fh_2) showed significant deviation from Hardy-Weinberg equilibrium. The corresponding F_{IS} value (Table 3.3) as well as the absolute numerical differences between expected and observed heterozygotes (data not shown) at this locus were low, so the deviation from Hardy-Weinberg equilibrium is minor. However, it should be remembered that the Hardy-Weinberg principle makes a number of assumptions which may not be upheld here including the fact that *F. hepatica* is capable of self-fertilisation (a form of inbreeding), and since we do not know the location of the microsatellites on the genome (Cwiklinski *et al.*, 2015a) there is the potential for these markers to be inherited with other genes under selection.

If two loci are linked they will be inherited together and this could affect the population structure detected. Therefore each pair of loci was assessed for evidence of linkage disequilibrium. Some pairs of loci showed significant *p*-values (Table 3.4) indicating they might be linked and inherited together. Therefore to determine the potential extent of linkage disequilibrium r^2, which gives an indirect indication of the physical distance between the loci pairs, was calculated (Table 3.4). All of the r^2 values were low indicating the pairs of loci are closer to equilibrium than disequilibrium. Furthermore, many of these pairs include loci which had large numbers of rare alleles (data not shown). The presence of rare alleles can artificially cause pairs of loci to appear linked. Therefore, taken together, none of the loci here are likely to be inherited together.

Table 3.4: Mean, median and range for r^2 as a measure of linkage disequilibrium for all pairs of loci that had significant *p*-values

Sample	Pair of loci	*p*-value*	r^2 median	r^2 mean	r^2 range
Parasites from sheep and cattle	Fh_6/Fh_10	0[12]	0.0001	0.0011	0 to 0.11
	Fh_6/Fh_12	0.00103[12]	0.0001	0.00083	0 to 0.11
	Fh_11/Fh_12	0[12]	0.0001	0.00098	0 to 0.027
	Fh_12/Fh_13	0.00439[2]	0.0001	0.00084	0 to 0.010
	Fh_5/Fh_15	0[12]	0	0.00096	0 to 0.17
Parasites from cattle	Fh_2/Fh_6	0.00432[12]	0.0001	0.0012	0 to 0.071
Parasites from sheep	Fh_2/Fh_6	0[12]	0.0001	0.00089	0 to 0.062
	Fh_5/Fh_6	0.00519[2]	0.0001	0.0011	0 to 0.025
	Fh_2/Fh_10	0.000026[12]	0.0001	0.0010	0 to 0.11
	Fh_6/Fh_10	0.0123[2]	0.0001	0.0014	0 to 0.061
	Fh_6/Fh_12	0[12]	0.0001	0.0017	0 to 0.33
	Fh_11/Fh_12	0.00279[2]	0.0002	0.0015	0 to 0.065
	Fh_6/Fh_13	0.00773[2]	0.0001	0.00098	0 to 0.029

* Bonferroni and false discovery rate correction were applied [1] = significant when Bonferroni correction applied ($p = 0.00179$) [2] = significant when false discovery rate correction applied

3.3.1 Do specific MLGs of Fasciola hepatica *aggregate in the definitive host?*

When the snail intermediate host is infected with one miracidia of *F. hepatica*, multiple genetically identical cercariae are produced: this is known as clonal expansion. This raises the possibility that the definitive host can be infected with multiple parasites of the same genotype. To determine if this occurs we identified the number of animals in which repeated MLG were detected (Figure 3.1). The majority of animals (61 %) contained a proportion of parasites with repeated MLG. More sheep (71 %) contained parasites with repeated MLGs than cattle (48 %) but this was not significantly different ($X^2 = 0.588$; $p = 0.4432$). In the majority of cases a repeated MLG was represented by two parasites (65 out of 96 repeated MLGs), and only one repeated MLG was seen (24 of 46 animals). In some hosts either a large number of parasites (nine or 10) with the same MLG were found, or multiple sets of repeated MLGs were observed in the same animal (20 out of 46 animals; maximum eight sets of repeated MLGs in one animal). There were also animals where each of the 36 parasites tested had a different unique MLG.

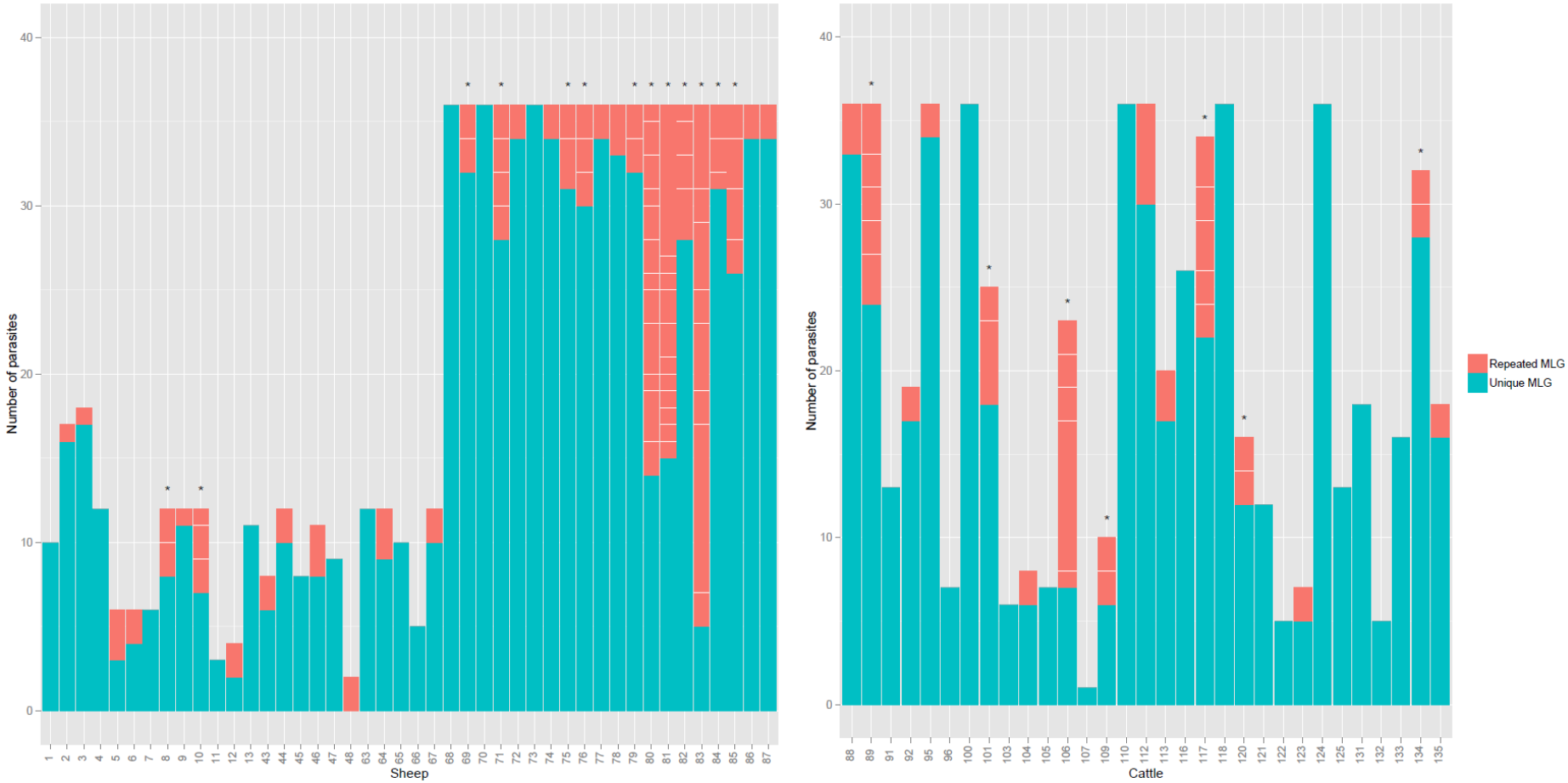

Figure 3.1: Representation of the number of parasites with repeated MLGs found within each individual sheep and cattle shown as a proportion of the total number of parasites genotyped from each definitive host; * indicate if more than one set of repeated MLGs was found in that individual host, the bar is split to distinguish the number of parasites within each set of repeated MLGs

In order to determine whether these repeated MLGs represented different reproductive events or were from the same clonal lineage P_{sex} values were calculated. All the P_{sex} values were highly significant at n = 2 and overall ranged from 1.74×10^{-71} to 0.00034 in parasites from sheep, and from 2.97×10^{-47} to 2.39×10^{-5} in parasites from cattle. Therefore all of the repeated MLGs detected represent clonal lineages. Furthermore, the minimum number of loci required to detect the maximum number of repeated MLGs was six. Since this is less than the eight loci used it means we have detected the maximum number of distinct MLGs (Arnaud-Haond *et al.*, 2005).

Generally parasites with repeated MLGs were found within the same animal, however parasites with repeated MLGs were also found shared between sheep (sheep 2 and 3; sheep 9 and 10; sheep 80 and 81; sheep 82 and 84) and between cattle (cattle 104 and 106), but were not found shared between sheep and cattle. Three of these pairs of animals (sheep 2 and 3; sheep 9 and 10; cattle 104 and 106) came from the same farms. The remaining two pairs came from the same geographical area, and potentially from the same farm, but this information was not available. In total 66 and 30 repeated MLGs, equating to 170 and 81 individual parasites (0.16 of all parasites), were identified in sheep and cattle, respectively. The number of parasites with repeated MLGs was significantly higher in sheep than cattle ($X^2 = 4.9052$; $p = 0.02678$) and this was not because parasite burdens tend to be higher in sheep since the burdens of the sheep and cattle sampled here were not significantly different (Mann-Whitney test $p = 0.5842$).

To determine whether repeated MLGs tended to co-occur in the same host Fisher's exact test was performed using a Monte Carlo simulation. When assessing all animals it was found that repeated MLGs did tend to co-occur in the same host ($p = 0.0002$). When assessing those animals that came from the same farms, it was found that repeated MLGs did tend to co-occur in the same host on three of the five sheep farms and one of the three cattle farms ($p < 0.00625$ using a Bonferroni correction). This suggests that at least in some instances there is clumped transmission of *F. hepatica* to the definitive host, and that substantial mixing of parasites with different MLGs does not occur prior to ingestion by the definitive host. The most likely explanation is that following clonal expansion in the snail and release of cercariae, metacercariae of the same genotype aggregate on pasture, and are then ingested by a sheep or a cow.

3.3.2 How much genetic diversity is there in the British Fasciola hepatica *population?*

Heterozygosity is a measure of genetic variation: the observed and unbiased heterozygosity for each locus are shown in Table 3.3. The heterozygosity of individual parasites ranged from 0.25 to 1. Therefore whilst there is individual parasite variation, the mean heterozygosity of all parasites across all loci was 0.752 (SD = 0.130), and this corresponds to high genetic variation.

Genotypic richness (R) is a measure of genetic diversity that describes the number of distinct MLG within a population. Here across all parasite samples the genetic diversity is high, R = 0.901. As with heterozygosity there is again individual variation, as the range of values for R within individual definitive hosts is 0.343 to 1. However, the majority of animals have a genotypic richness of greater than 0.8 (Figure 3.2).

The Pareto distribution (Figure 3.3) is a graphical representation of the richness (i.e. the number of distinct MLGs) and the evenness (i.e. the distribution of the MLGs across the definitive hosts studied). Here we can see that since r^2 values are high and *p*-values are significant the distribution of MLGs does follow the Pareto distribution. The distribution of MLGs is highly skewed: most MLGs are unique, with much fewer parasites being represented by repeated MLGs. The value of β (the slope of the fitted log-log regression equation) is high in all cases. This means that there is a high evenness with clonal lineages being approximately comparable in size. The values for β are similar when assessing parasites from sheep and cattle separately, and no significant difference was found between the MLG distribution in sheep and cattle using a linear model ($p = 0.325$). Overall the British *F. hepatica* population has high genetic diversity, with a small proportion of parasites (0.16) being genotypically identical.

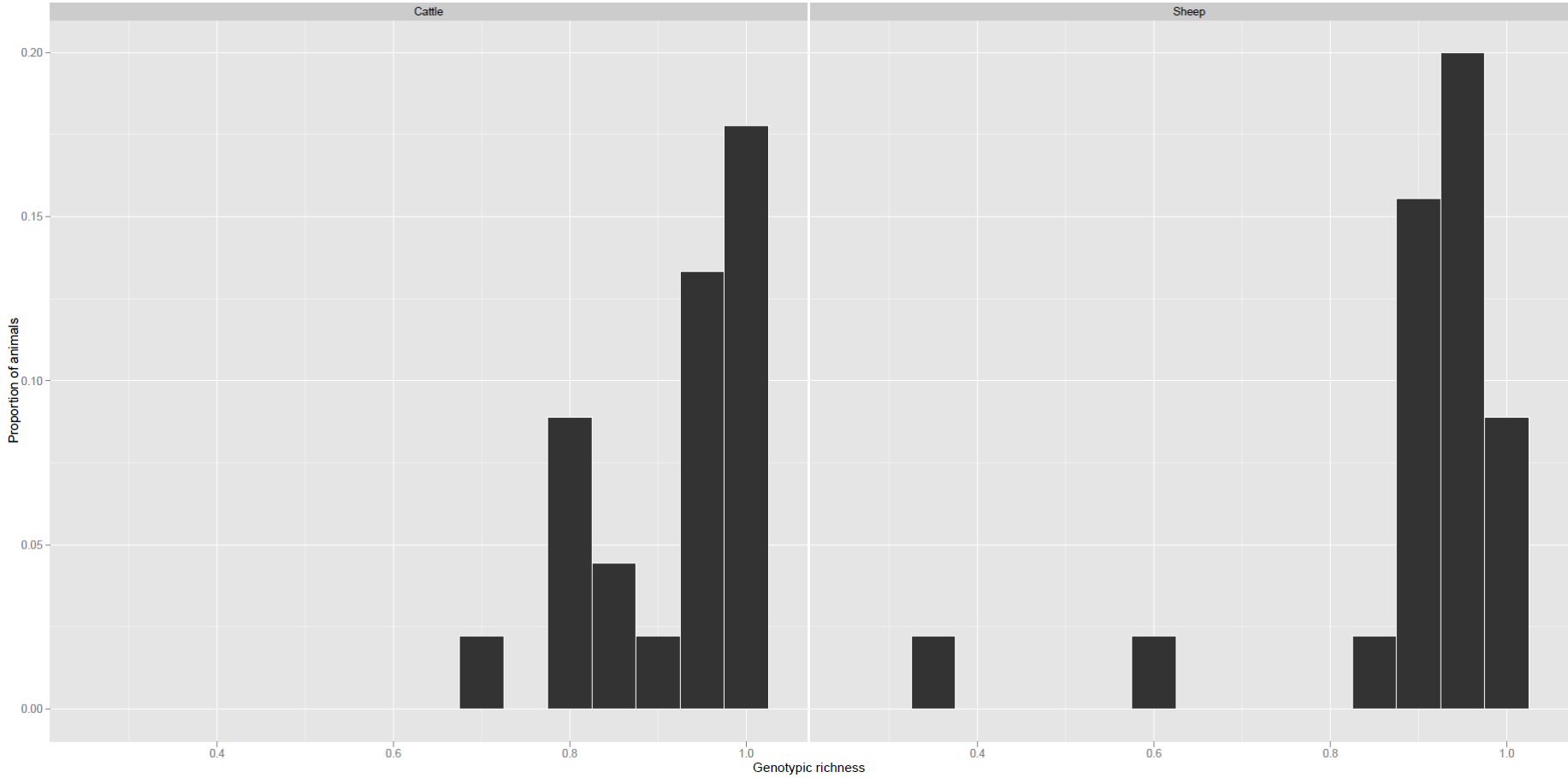

Figure 3.2: Histogram displaying the genotypic richness values within each definitive host, separated into cattle and sheep

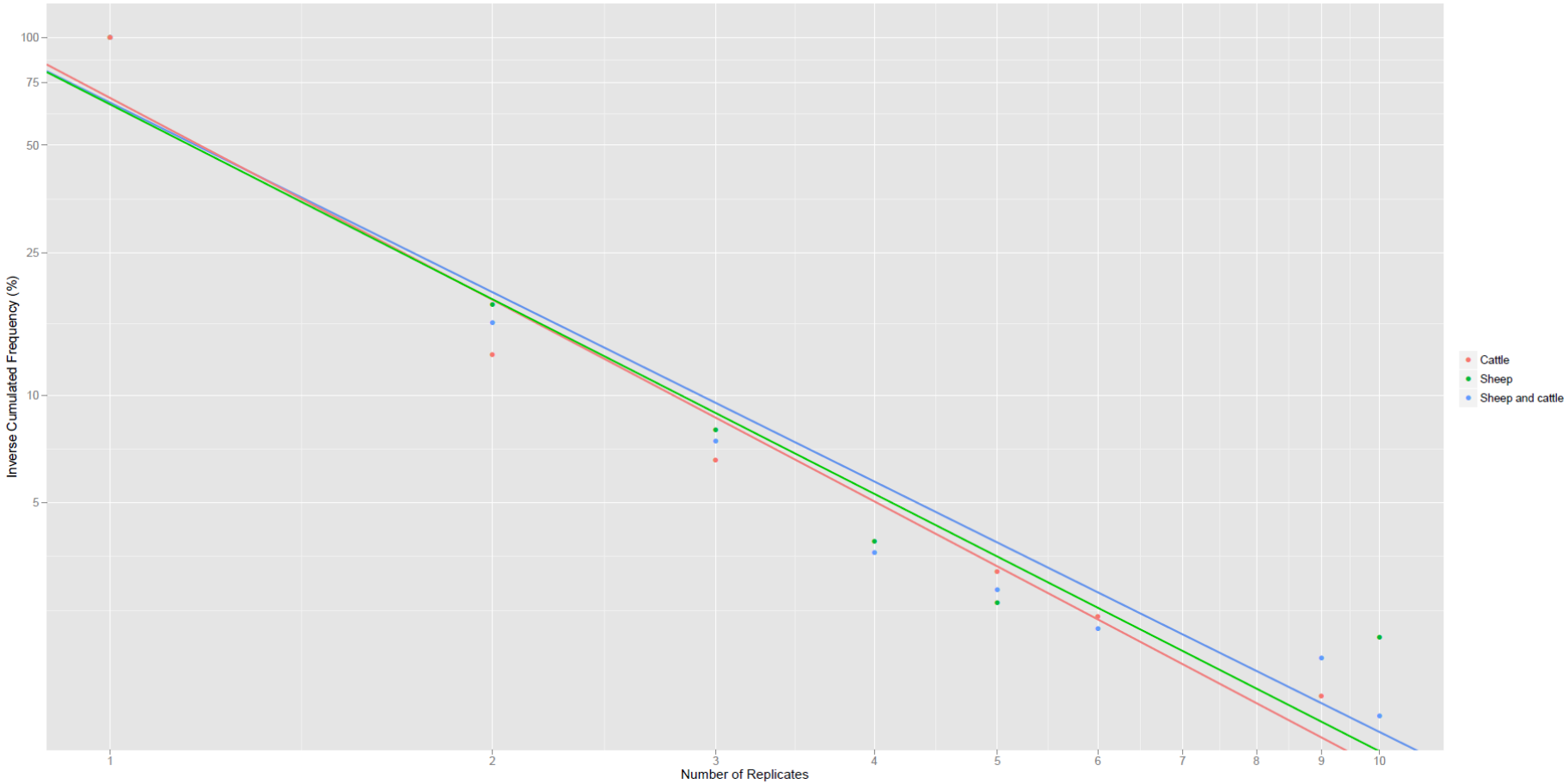

Figure 3.3: Graphical representation of genotypic richness and evenness; **Cattle**: $\beta = 2.87$, $r^2 = 0.964$, $p = 0.000504$; **Sheep**: $\beta = 2.76$, $r^2 = 0.916$, $p = 0.00270$; **Sheep and cattle**: $\beta = 2.81$, $r^2 = 0.953$, $p = 3.23 \times 10^{-5}$

3.3.3 Are Fasciola hepatica *from sheep and cattle genetically different?*

We have seen that when assessing the Pareto distribution there was no statistical difference between the distribution of repeated MLGs within sheep and cattle. There is no statistical difference in the level of genetic variation and diversity when parasites from sheep and cattle are assessed separately since (i) heterozygosity across all loci is 0.758 (SD: 0.141) in sheep and 0.745 (SD: 0.118) in cattle, no significant difference (Mann-Whitney $p = 0.092$); and (ii) the genotypic richness across all parasites is 0.890 in sheep and 0.918 in cattle, no significant difference (Mann-Whitney $p = 0.689$).

Although the level of genetic variation is similar between sheep and cattle, it was also necessary to establish whether parasites from sheep and cattle shared the same alleles. Sheep and cattle have a number of alleles and genotypes in common across all loci (data not available for locus Fh_1). Private (unique) alleles were identified when comparing parasites from sheep and cattle: 14.7 % and 6.0 % of all alleles were unique to sheep and cattle, respectively. The most common allele at each locus was usually identical (differing only at loci Fh_2 and Fh_4, data not available for locus Fh_1) when comparing parasites from sheep and cattle. The most common genotypes were also identical at nine loci (Fh_5, Fh_7, Fh_8, Fh_9, Fh_10, Fh_11, Fh_12, Fh_14 and Fh_15). Therefore parasites from sheep and cattle show not only a similar level of genetic variation, but also largely similar alleles and genotypes. Although private alleles were identified when comparing parasites from sheep and cattle it is possible that if more parasites were genotyped more alleles in common might be found.

F_{ST} gives a measure of genetic differentiation, i.e. if it is low, populations are genetically similar. The pairwise F_{ST} between parasites from sheep and cattle was 0.00145 and was statistically significant ($p < 0.05$). This value is low indicating little genetic differentiation and therefore means the parasites from sheep and cattle are genetically similar. Furthermore PCA analysis of pairwise F_{ST} values between the parasites within each definitive host does not reveal any clustering based on species (Figure 3.4). Therefore, from the evidence presented in this study there does not appear to be structuring of the parasites from sheep and cattle, and *F. hepatica* from the two species of definitive host are genetically similar.

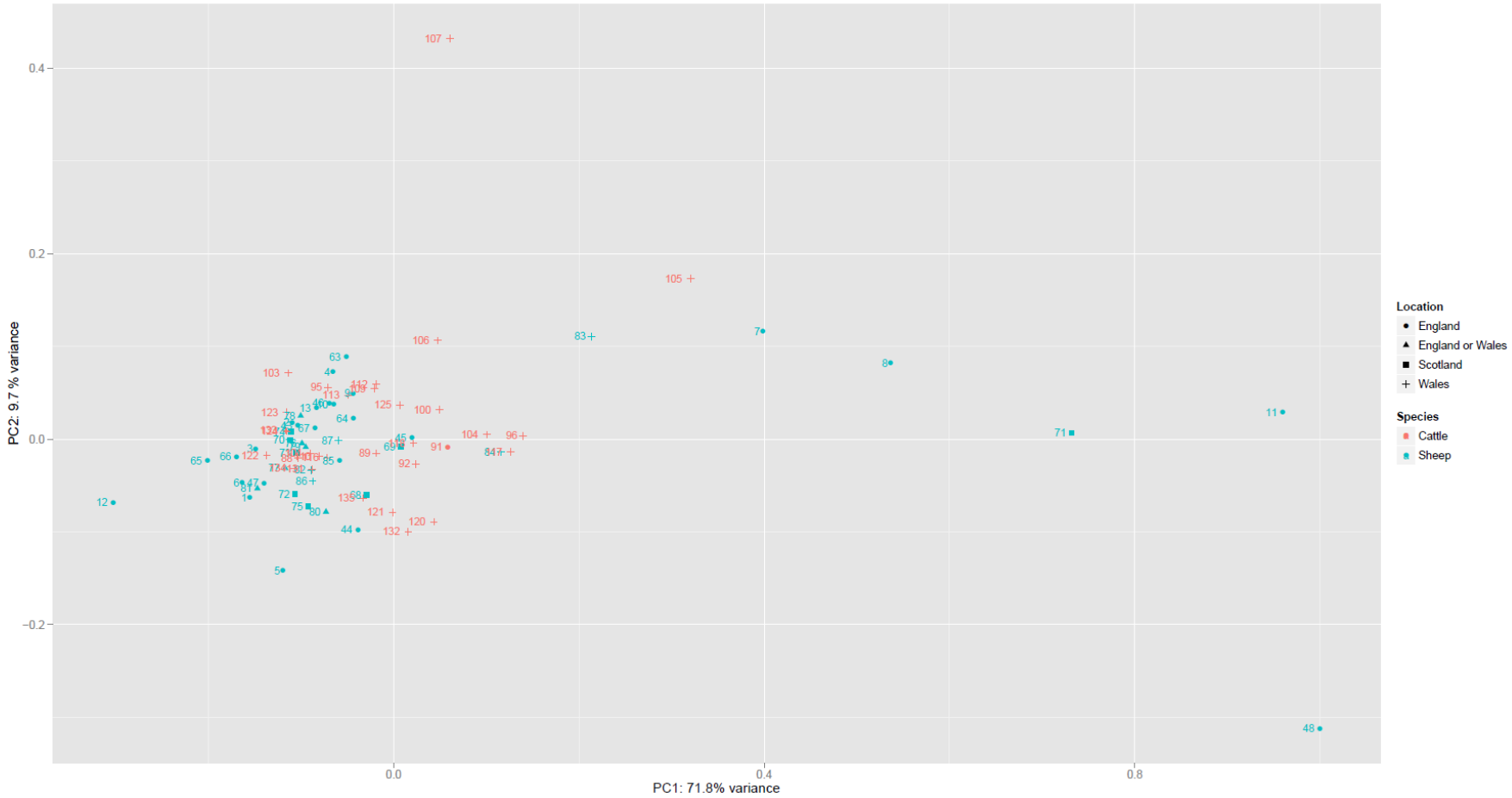

Figure 3.4: Principle component analysis for pairwise F_{ST} values between the parasites of the different definitive hosts and coded based on species and location information

3.3.4 Is the British Fasciola hepatica *population panmictic or is there geographical structuring?*

We have seen in Figure 3.4 that based on PCA analysis of pairwise F_{ST} there is no clustering based on species. There is also no clustering based on the location of the definitive host (Figure 3.4), thereby indicating that there is no variation in the level of genetic differentiation across Britain. It is important to check for isolation by distance since there can appear to be population structure in populations that are actually isolated by distance. Exact location information was available for cattle only and based on ear tag data. There was no evidence of isolation by distance since the slope of the regression line was negative, and the *p*-value was non-significant (Figure 3.5).

Structure 2.3.4 (Pritchard *et al.*, 2000) is able to detect subtle population structure. The results from Structure (Pritchard *et al.*, 2000) are shown in Figure 3.6. The mean natural log probabilities (Figure 3.6A) do not appear to reach an asymptote, therefore indicating a single population with no structure. The majority of ΔK values are low (Figure 3.6B) but results from K equal to 7 and 45 were assessed in more detail (not shown): the majority of the individual parasites were assigned to each population in equal proportions indicating no population structure (Pritchard *et al.*, 2010). Phylogenetic trees can also give an indication of clusters of parasites. A neighbour-joining tree was created and bootstrapped 1000 times (not shown). However the majority of nodes had bootstrap values of less than 40 % indicating that the topology of the tree is not reliable enough from which to draw conclusions. Finally by looking at F_{ST} between definitive hosts (across all parasites and loci) we get an indication of population structure. F_{ST} was 0.0202, this value is low which indicates little genetic differentiation and also low population structure. Therefore the evidence suggests that the British *F. hepatica* population is a single panmictic population with no geographical structuring.

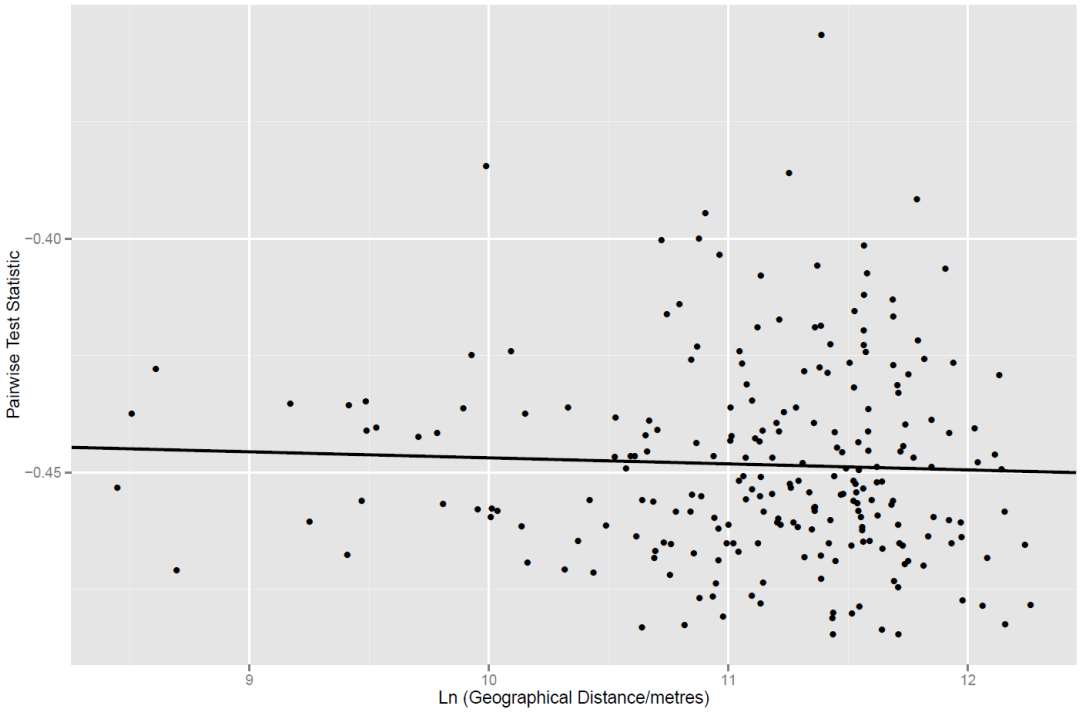

Figure 3.5: Results from isolation by distance with cattle parasites grouped according to farm; pairwise test statistic is based on F_{ST} / (1-F_{ST}); regression line: slope = -0.00129 (95 % CI: -0.00317, 0.00142), intercept = -0.434, *p*-value = 0.2968

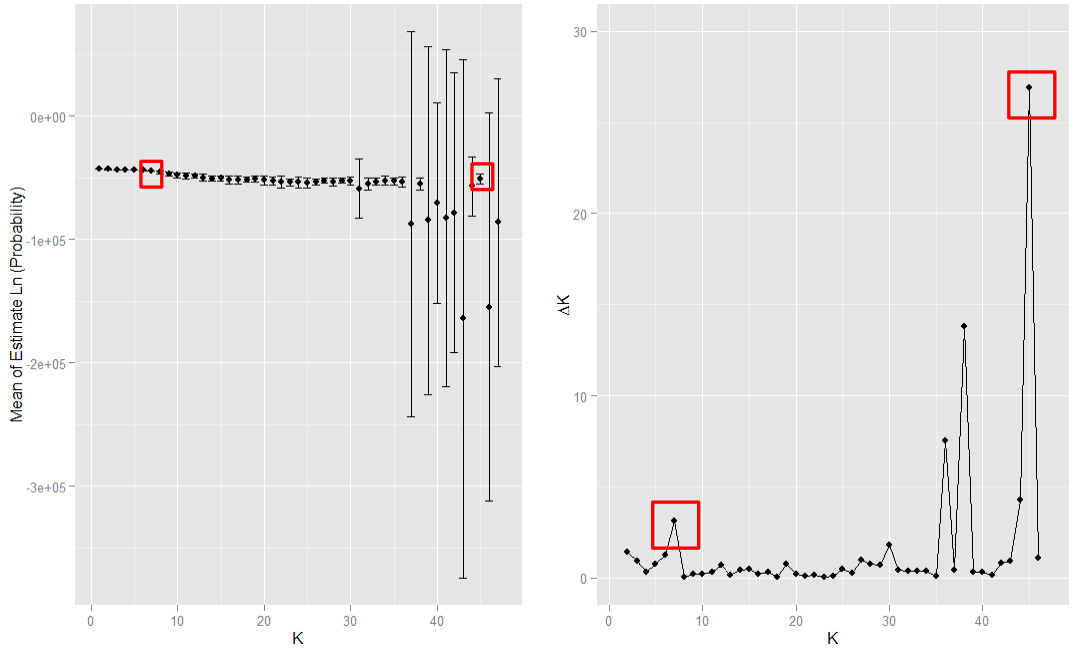

Figure 3.6: Results to assess for population structure calculated using Structure (Pritchard *et al.*, 2000) and Structure Harvester (Earl and vonHoldt, 2012); each value of K is plotted against **A**: the mean natural log probabilities with error bars showing standard deviations (Pritchard *et al.*, 2000) and **B**: ΔK (Evanno *et al.*, 2005); red boxes highlight K = 7 and K = 45 on each graph

3.3.5 What is the level of gene flow in the British Fasciola hepatica *population?*

F_{ST} gives not only an indication of population structure and genetic differentiation, but can also be used to infer gene flow. The F_{ST} between definitive hosts is 0.0202 and since this indicates little genetic differentiation, this means high gene flow in the population. This fits with the evidence already presented that the British *F. hepatica* population is panmictic. When parasites from sheep and cattle were assessed separately the F_{ST} values between sheep and between cattle were very similar: 0.0193 and 0.0207, respectively.

Since private alleles were identified, N_m (the effective number of migrants) can be used to give an indirect estimate of gene flow. If gene flow is high there is a higher probability that a migrant will enter the population with a rare allele (Slatkin, 1985). As this method was developed using only sample sizes less than 100, it might not hold when sample size is large. Therefore parasites were grouped based on the definitive host they came from giving a mean sample size of 18.99. N_m across all loci was 5.59, and since this is greater than 2, it is indicative of high gene flow (Slatkin, 1985). When parasites from sheep and cattle were assessed separately, N_m values were 6.85 and 8.20, respectively.

Therefore both the F_{ST} and N_m values support that there is high level of gene flow in the British *F. hepatica* population. F_{ST} reports a slightly higher level of gene flow in parasites from sheep than cattle, whereas N_m reports the opposite, so gene flow is probably similar in the two definitive host species, and genes flow freely between sheep and cattle.

3.3.6 How much self-fertilisation occurs in the British Fasciola hepatica *population?*

Being a hermaphrodite, *F. hepatica* is capable of self- and cross-fertilisation. Since self-fertilisation is in effect a form of inbreeding, F_{IS} values, the inbreeding co-efficient, can be used to determine the rate of self-fertilisation (s) using the equation

$$F_{IS} = s / (2-s)$$

F_{IS} values in parasites from cattle are greater than zero (0.0115), but in sheep are less than zero (-0.0060) indicating that in cattle there is more inbreeding than expected and in sheep there is less inbreeding than expected. The F_{IS} across all loci and parasites is 0.0011 (95 % CI: -0.011, 0.013). This is indistinguishable from zero and means the selfing rate is no higher than 2 %.

3.4 DISCUSSION

3.4.1 The British Fasciola hepatica *population is genetically diverse and panmictic*

The *F. hepatica* collected here are from naturally infected sheep and cattle representing populations from Scotland, England and Wales. We have shown that the British *F. hepatica* population is genetically diverse and panmictic, with no evidence of sub-structuring amongst parasites from sheep and cattle, or geographically across Great Britain. Similarly studies of *F. hepatica* from Spain using allozyme markers (Vázquez-Prieto *et al.*, 2011), and from Bolivia using five microsatellites markers (Hurtez-Boussès *et al.*, 2004), have also found evidence of a panmictic parasite population.

High variation and diversity is commonly reported in population genetic studies of *F. hepatica* from other countries including: haplotype diversity of 0.832 based on the *NAD1* gene and 0.482 based on the *COX1* gene in parasites from Australia (Elliott *et al.*, 2014); a mean haplotype diversity of 0.76 based on mitochondrial markers in parasites from eastern Europe and western Asia (Semyenova *et al.*, 2006); and a mean haplotype diversity of 0.836 from European *F. hepatica* samples based on both nuclear and mitochondrial sequences (Walker *et al.*, 2012). Whilst these studies have reported high diversity, the absolute number of haplotypes reported is low: using two mitochondrial markers 20 composite haplotypes from 208 parasites were found (Elliott *et al.*, 2014), and using three mitochondrial markers 52 composite haplotypes from 221 parasites were found (Walker *et al.*, 2007). Novel haplotypes were found from one year to the next in the Netherlands based on mitochondrial markers (Walker *et al.*, 2011), showing the potential for *F. hepatica* to maintain and increase its diversity. It has been noted by Zintl *et al.*, (2015) that parasites with higher heterozygosity at some microsatellite loci were more likely to establish in the liver following infection, compared to those with a lower heterozygosity. Maintenance of this high variation may be a selective advantage. Here, our study was able to identify a greater number of MLG. This is likely to be due to the use of microsatellites which are known to be distributed throughout the *F. hepatica* genome (Cwiklinski *et al.*, 2015a) and highlights the superiority of a microsatellite-based approach, as supported by others (Vilas *et al.*, 2012).

In our study the genotypic richness across all parasites was 0.901 (0.890 in parasites from sheep, and 0.918 in parasites from cattle). Using the same measure the study by Vilas *et al.*, (2012) showed genotypic richness across all parasites of 0.678 (0.477 in parasites from sheep, and 0.747 in parasites from cattle). Therefore a greater genotypic diversity has been identified in British populations of *F. hepatica* compared to Spanish populations. This could, in part, be due to the increased number of microsatellite markers used in this study compared to by Vilas *et al.*, (2012). In both studies there are examples where all the parasites within one definitive host were found to have distinct MLGs. Similar findings have been reported by Semyenova *et al.*, (2006) but with far smaller sample sizes; and in animals from Australia up to 10 different mitochondrial haplotypes were found in a single host (Elliott *et al.*, 2014).

Our study reports low F_{ST} indicating low population structure. Similar findings have also been reported elsewhere in Europe: F_{ST} was 0.006 in Spanish *F. hepatica* (Vázquez-Prieto *et al.*, 2011), and 0.0228 in populations of *F. hepatica* from the Netherlands (Walker *et al.*, 2011). The majority of genetic variation is therefore seen within parasites in the definitive host, and only a small amount of variation is due to differences between these hosts. Whilst in our study similar F_{ST} values were reported in sheep and cattle, an F_{ST} value of 0.170 was reported in parasites from sheep by Vilas *et al.*, (2012). This was approximately double the value reported for cattle and is indicative of substantial genetic differentiation. This is interesting since the sheep studied were all from the same farm where one would expect little genetic differentiation, and is in contrast to the F_{ST} described in cattle which came from multiple farms. The authors suggest the difference may be caused by the practice of feeding cattle on multiple sources of pasture compared to sheep; but the effect of low parasitic load in sheep (median burden of 11 parasites) may also influence the structure seen (Vilas *et al*, 2012). Small, distinct snail habitats with restricted grazing, or buying in of animals with genetically novel parasites may also play a role.

We found no evidence of geographical structure and no difference between parasites collected from sheep compared to cattle. The bootstrap values for the phylogenetic trees constructed (not shown) indicated that the topology of the tree was unreliable. This is likely to be a product of using a limited number of microsatellite loci, since computer simulations have suggested 30 microsatellites

may be required to determine accurate evolutionary patterns (Koskinen *et al.*, 2004). Therefore analysis of further loci in the future could reveal structure not previously detected. We do not know if any of the sheep and cattle sampled in this study were co-grazed with, or grazed on pasture used by, the other species. If sheep and cattle share the same pastures this gives a population of *F. hepatica* parasites the opportunity to infect both species, giving the appearance of panmixia between sheep and cattle. In a study of 606 British dairy farms, approximately 25 % also had sheep on farm at some point during the year (Personal Communication Ms A. Howell). This does not definitively mean the animals shared the same pastures, and would be unlikely to apply to all the animals sampled here. However even a small amount of migration is enough to destroy any observed population structure and emulate panmixia.

A lack of population structure in relation to location and definitive host species has also been reported in *F. hepatica* from Spain using SRAP markers (Alassad *et al.*, 2008), and in eastern European and western Asian *F. hepatica* based on sequencing of mitochondrial genes (Semyenova *et al.*, 2006). It has also been shown that *F. hepatica* populations from sheep and cattle from the same farm had a low F_{ST} value (0.00609) indicating little genetic differentiation (Elliott *et al.*, 2014). Therefore in areas where sheep and cattle are exposed to the same parasite the population structure is similar in the two species, and supports our findings. Conversely previous studies have found differences in RAPD patterns from *Fasciola* spp. from different mammalian hosts (Vargas *et al.*, 2003; Ramadan and Saber, 2004; Aldemir, 2006). However, analysis of *F. gigantica* using the same technique revealed no difference between parasites from sheep and cattle (McGarry *et al.*, 2007). We, and others, have shown that *F. hepatica* populations are genetically diverse and variable. Therefore, these different RAPD patterns may be due to technical genetic variation rather than differences between parasites from distinctive species. It should also be remembered that there is concern over the reproducibility of RAPD banding patterns (Ellsworth *et al.*, 1993).

In this study we have shown that adult *F. hepatica* in the definitive host are genetically diverse. The sheep sampled in this study were lambs that had grazed for only one season, yet they displayed highly diverse adult parasite populations. This suggests that the metacercariae on pasture, to which the lambs were exposed, were also highly genetically diverse. Similar findings have been reported by Walker *et al.*,

(2007) where one calf which had grazed for only one or two seasons had 10 mitochondrial haplotypes represented in the parasites recovered. The ability of *F. hepatica* to maintain this level of genetic diversity despite the fact that each generation must pass through the snail intermediate host is interesting. Studies in other trematode parasites have found population sub-structuring in animals that play only a minor role in transmission (Wang *et al.*, 2006). Therefore there is the potential that other species of animals in Great Britain, for example hares and rabbits, which play only a minor role in transmission, may contribute to the genetic diversity on pasture and ultimately in the definitive host.

The British *F. hepatica* population is panmictic and there is no evidence of geographical structuring. This is a feature common to many nematode species of livestock when studied across a single country (Anderson *et al.*, 1998). However in *Teladorsagia circumcincta* some evidence of genetic differentiation between parasites from different countries was observed (Grillo *et al.*, 2007). There is also evidence of geographical structuring of *Fascioloides magna* across North America; this is thought to be related to the uneven distribution of the major definitive host (deer) and the lack of movement of these wild animals compared to livestock (Bazsalovicsová *et al.*, 2015). It would be interesting to identify the level of genetic diversity in, and genetic differentiation between, populations of *F. hepatica* from wild, as opposed to farmed, definitive hosts, as well as populations of *F. hepatica* from different countries. This would be particularly interesting in countries where *F. hepatica* co-exists with *F. gigantica* and where hybridisation between the two species is known to occur (see Chapter 2).

Other parasites of livestock have also been shown to have a high genetic diversity including *Teladorsagia* spp. and *Haemonchus contortus* from sheep; and *Ostertagia ostertagi* and *Haemonchus placei* from cattle. In line with our findings in *F. hepatica*, most of the diversity exists within the definitive host; it is suggested this may be due to the large effective population size of parasites that exists in any one definitive host (Blouin *et al.* 1995; Anderson *et al.*, 1998; Braisher *et al.*, 2004; Grillo *et al.*, 2007). However, although burdens of *F. hepatica* vary between hosts they are unlikely to reach the same levels as the gastrointestinal nematodes which can number in the thousands, and every generation of *F. hepatica* must pass through the snail intermediate host. Therefore there are likely to be other factors that maintain the level of genetic diversity seen in *F. hepatica*.

3.4.2 Gene flow in the British Fasciola hepatica *population is high*

The F_{ST} values reported here and by other studies are low (Vázquez-Prieto *et al.*, 2011, Walker *et al.*, 2011; Vilas *et al.*, 2012), which along with low population structure also indicates high gene flow. Our study showed an F_{ST} value that was higher in parasites from cattle, whilst a study in Spain found an F_{ST} value in parasites from sheep that was almost double that observed in parasites from cattle (Vilas *et al.*, 2012). It is suggested this is because in Spain, cattle have more opportunity to graze from different pastures compared to sheep, which may affect gene flow (Vilas *et al.*, 2012). In other livestock parasites, *Teladorsagia* spp. and *H. contortus* from sheep, and *O. ostertagi* and *H. placei* from cattle; high gene flow is also observed (Blouin *et al.* 1995; Braisher *et al*., 2004). This is in contrast to *Mazamastrongylus odocoilei*, a parasite of deer, which showed lower levels of gene flow, isolation by distance and population structure (Blouin *et al.*, 1995). Therefore it is suggested that movement of the host, as a consequence of farming, determines the parasite population structure (Blouin *et al.*, 1995). Although the nematodes on which this hypothesis is based have simple one host life cycles, it is unlikely that the intermediate host of *F. hepatica* is able to migrate sufficiently to influence the population genetic structure. In addition any migration associated with the intermediate host is likely to be accidental, for example on farming equipment, and on a small scale. It has already been suggested that movement of the definitive host is an important factor in maintaining high levels of gene flow in *F. hepatica* (Semyenova *et al.*, 2006).

3.4.3 Cross-fertilisation predominates in the British Fasciola hepatica *population*

In our study the selfing rate estimated from F_{IS} values is low, however the F_{IS} values reported by Vilas *et al.*, (2012) would give selfing rates of 35.6 % and 42.6 % in sheep and cattle, respectively: much higher than reported here. It is suggested that since there are more parasites with repeated MLGs in sheep there is the potential for more inbreeding (Vilas *et al.*, 2012), but as selfing is a form of inbreeding this does not hold since the selfing rate in sheep is lower than cattle (both including and reducing repeated MLGs). F_{IS} in cattle was also higher in our study, and so there may be a tendency for parasites in cattle to self-fertilise more than in sheep. Cattle livers are extremely large compared to sheep, and their parasite burdens tend to be lower than in sheep. Therefore the likelihood of parasites meeting each other to reproduce may be reduced in cattle livers, and so, as reported here, you might expect higher

levels of self-fertilisation in cattle compared to sheep. Regardless the self-fertilisation rate is still very low in the British *F. hepatica* population. Differences in the levels of self- and cross-fertilisation have been noted in our laboratory between different isolates of *F. hepatica* (Hodgkinson *et al.*, unpublished) and in *Schistosoma mansoni* levels of inbreeding have been related to the age, and therefore immunity, of the definitive host (Van den Broeck *et al.*, 2014). Therefore in some instances the level of selfing could be higher.

3.4.4 Hosts harbour repeated MLGs and Fasciola hepatica *shows evidence of clumped transmission*

The majority of animals in this study contained parasites which had repeated MLGs. Repeated MLGs could be represented by two parasites or more than two parasites, and as P_{sex} values were significant at n = 2 each set of repeated MLGs arose from the same clonal lineage rather than distinct reproductive events. Similarly the majority of animals (17 of 20) studied by Vilas *et al.*, (2012) contained parasites with repeated MLGs, some MLGs were represented by large numbers of parasites, and some animals contained numerous sets of repeated MLGs. However, two of the repeated MLGs found in sheep had P_{sex} values that were significant at n = 3, rather than n = 2; indicating it is likely that one parasite has arisen from a distinct reproductive event whilst the remainder are from the same clonal lineage (Vilas *et al.*, 2012). In contrast to our study, repeated MLGs in parasites from Spanish sheep led to structuring of the population, but all of the sheep genotyped were from the same farm (Vilas *et al.*, 2012).

We found parasites with repeated MLGs shared between animals from the same geographical areas; and, where we had the information, this was known to be the same farm. In addition to observing the same phenomenon Vilas *et al.*, (2012) also report one repeated MLG that was shared between two cattle from distinct geographical areas; this may be related to the movement of animals from different areas. Neither study report MLGs shared between sheep and cattle, but this could be related to co-grazing: if parasites were collected from sheep and cattle that were known to co-graze the same pastures, MLGs may be found shared between them.

Since repeated MLGs tended to co-occur in the same animal there is evidence of clumped transmission of parasites in our study, which is consistent with the findings of Vilas *et al.*, (2012). In our study this was not consistent across all the

farms studied. This may be related to the size of the snail habitat, which might influence how large an area of pasture metacercariae are found on. Little information is available on how far snails roam, or the distance cercariae move from the snail before encysting. If a snail habitat is small, the distribution of cercariae from different snails may overlap on the pasture before transmission to the definitive host. If snails are infected with miracidia of more than one genotype, and shed cercariae of more than one genotype at the same time, there may be clumped transmission of more than one genotype.

In both our study, and the study by Vilas *et al.*, (2012), more parasites with repeated MLGs were found in sheep than cattle. This could be related to the grazing patterns of sheep compared to cattle, or differences between the immune systems of the two species that affect the proportions of metacercariae that successfully excyst and migrate to the liver.

3.4.5 Conclusion

The British *F. hepatica* population shows high gene flow and we have identified a proportion of parasites within the definitive host that are genetically identical. The level of genetic diversity and genetic variation in the British *F. hepatica* population is high within populations and there is little genetic differentiation between populations, indicating panmixia.

We know that every generation of *F. hepatica* must pass through the snail intermediate host, which in effect creates a population bottleneck. It is interesting to speculate how the parasite is able to maintain the level of genetic diversity seen both on pasture and in the definitive host. Since the sheep in this study had a similar genetic diversity to cattle, yet had achieved this over one grazing season, diversity is not just a product of grazing over time. Although *F. hepatica* are highly fecund, this would still have to be translated through the snail to help in the maintenance of genetic diversity. If a snail can be infected with more than one genotype of *F. hepatica* and these all undergo clonal expansion, this could give the parasite the potential to overcome the bottleneck imposed by the snail.

CHAPTER 4

How does the snail intermediate host influence genetic variation and gene flow in *Fasciola hepatica* populations?

4.1 INTRODUCTION

The presence of the intermediate host is essential to the transmission of *Fasciola hepatica*. In most areas of the world where *F. hepatica* exists, the intermediate host is the mud snail, *Galba truncatula* (Boray, 1969). Knowledge of snail habitats, species of snails acting as intermediate hosts, and prevalence of *F. hepatica* infection within the snail are important when considering the epidemiology of this parasite (Canete *et al.*, 2004). Ultimately a snail will only drive parasite transmission if it sheds cercariae. A number of factors (e.g., the definitive host from which eggs and miracidia originate, and nutrition, age, or size of the snail) can influence the capacity of the snail to produce cercariae. The relatively low pathogenic effect of *F. hepatica* on the snail intermediate host means snails can survive for a long time and produce cercariae over a prolonged period. Whilst the rapid clonal expansion that occurs within the snail, means the parasite can produce numerous cercariae (Boray, 1978), and shed several hundred cercariae at a time (Hodasi, 1972).

To appreciate how passage of the parasite through the snail affects population structure it is necessary to understand the prevalence of *F. hepatica* infection within the snail and the genetic variation of parasites within the snail by sampling snails from the field. *Galba truncatula* is usually found in semi-aquatic habitats (Boray, 1969) including drainage furrows, slow moving streams and river banks (Schweizer *et al.*, 2007; Rondelaud *et al.*, 2011). However it can be difficult to identify snail habitats on farms especially considering that primary snail habitats are usually less than 5 m^2 (Rondelaud *et al.*, 2011). Other species of snail are reported to sustain development of *F. hepatica* (see Chapter 1); therefore accurate identification of snail species is important. Definitive species identification of the Lymnaeidae family is problematic largely due to conflicting species identification by morphological and molecular techniques, and redundant nomenclature. Morphological differentiation between species is clearest in European Lymnaeidae snails compared to other continents (Bargues and Mas-Coma, 1997), and the ITS-2 region can be used for molecular differentiation (Bargues *et al.*, 2001; Bargues and Mas-Coma, 2005).

Although infection of snails with *F. hepatica* can be detected by non-molecular methods they are time consuming and molecular techniques have been

developed to detect *F. hepatica* within snails; the most widely used are based on PCR (Kaplan *et al.*, 1995; Caron *et al.*, 2007; Novobilsky *et al.*, 2013). Other PCRs to detect *F. hepatica* DNA exist but have not been used to detect infection within snails (see Chapter 1). PCR based methods have been found to be more sensitive than microscopic methods (crushing snails to detect the intra-molluscan stages of the parasite, or dissection of snails; Caron *et al.*, 2007; Relf *et al.*, 2011). The most recent prevalence information of *F. hepatica* infection within *G. truncatula* in the UK and the Republic of Ireland is based on PCR and showed that in Wales overall 13.4 % of 134 snails were positive for *F. hepatica* and in the Republic of Ireland overall 13.8 % of the 974 snails tested were positive for *F. hepatica*, although in both studies this varied depending on the season (Relf *et al.*, 2011; Jones *et al.*, 2015). However, the only method that gives an indication of the true transmission potential of the snail, and thus the ability of the snail to affect the population structure of the parasite, is to observe shedding of cercariae.

The first aim of this chapter was to determine whether a snail can be infected with, and produce cercariae of, more than one genotype. This was assessed by performing experimental infections of snails, from a laboratory maintained colony of *G. truncatula*, with *F. hepatica* clonal isolates. Six clonal isolates, available at the University of Liverpool, were genotyped using the microsatellite panel described in Chapter 3, and isolates with distinct genotypes were used for the experimental infections. Following exposure to miracidia, the snails were maintained in the laboratory, and evaluated for shedding of cercariae. Microsatellite profiling was used to determine the genotype of infection within the snail, and of cercariae that were produced.

The second aim was to evaluate the population structure of *F. hepatica* within field populations of *G. truncatula*. Snails were collected from farms with known *F. hepatica* infection in their livestock. Snails were morphologically identified and this identification confirmed by PCR and sequencing of the ITS-2. Three methods were used to evaluate snails for *F. hepatica* infection (i) monitoring shedding of cercariae, (ii) crushing and visualisation of intra-molluscan stages under the microscope, and (iii) molecular identification by PCR. Microsatellite genotyping was performed on any cercariae produced, and on any snails positive for *F. hepatica* infection.

4.2 MATERIALS AND METHODS

4.2.1 Extraction of DNA from snails

Two commercially available DNA extraction kits were evaluated the DNeasy Blood & Tissue Kit (Qiagen) and NucleoSpin Tissue Kit (Macherey-Nagel, Fisher Scientific, Loughborough, UK). All reagents were included in the kits except for molecular grade ethanol (Sigma-Aldrich). The kits were used as per manufacturers' instructions with the following modifications: (i) prior to lysis, snails were macerated using a bead beater (Biospec Products Mini Beadbeater, Oklahoma, USA) with three times the specified volumes of buffers (ATL or T1) and proteinase K, and a 1:1 ratio of 0.1 and 1 mm glass beads. Two 30 second steps at 4800 oscillations per minute were performed with the sample placed on ice in between, (ii) samples were incubated at 56^{o}C overnight, (iii) three times the specified volumes of buffers (AL or B3) and 100 % molecular grade ethanol were used, and (iv) when transferring the mixture to the spin column, due to the increased volume, this step had to be performed multiple times – the same spin column was used and the flow through was discarded each time. DNA was stored at 4^{o}C.

These two methods were evaluated by quantifying the concentration of DNA produced from three individual snail extractions per kit. DNA was quantified using the Quant-it Pico Green dsDNA Assay Kit (Life Technologies) as described in section 3.2.3. The mean DNA concentration from the Qiagen DNeasy Blood & Tissue Kit was 113.6 ngµl^{-1} and from the Macherey-Nagel NucleoSpin Tissue Kit was 71.1 ngµl^{-1}. Therefore the Qiagen DNeasy Blood & Tissue Kit was used for all subsequent DNA extractions from snails. There was variation in the size of snails collected from the field (see 4.2.7): *G. truncatula* and *Potamopyrgus jenkinsi* were small (< 4 mm) and the mean concentration of DNA from each was 4.1 ngµl^{-1} and 12.3 ngµl^{-1}, respectively. In comparison the *Radix balthica* and *Succinea* spp. collected were larger (> 7 mm) and the mean concentration of DNA from each was 92.25 ngµl^{-1} and 65.15 ngµl^{-1}, respectively.

4.2.2 Extraction of DNA from trematode cercariae or metacercariae

The Qiagen DNeasy Blood & Tissue Kit was used as per manufacturer's instructions with the same modifications as described for snails (see 4.2.1) except that five times the volumes stated for ATL, proteinase K, AL and molecular grade ethanol were used. In addition the bead beater protocol was two 1 minute steps at 4800 oscillations per minute with the sample placed on ice in between. Elution was with 60 µl of AE.

4.2.3 Molecular identification of snails by ITS-2 PCR

Molecular identification of snails was performed using a PCR to amplify the ITS-2 region (referred to as Bargues ITS-2). Primers were as previously described for amplification of the ITS-2 from Lymnaeidae snails: LT1 (forward primer) 5' – TCG TCT GTG TGA GGG TCG – 3' and ITS-2 RIXO (reverse primer) 5' – TTC TAT GCT TAA ATT CAG GGG – 3' (Almeyda-Artigas *et al.*, 2000; Bargues *et al.*, 2001). PCR reaction was as described by Novobilsky *et al.*, (2013) with the following modifications (i) Biomix Red (Bioline, London, UK) was used as the PCR reaction mix, and (ii) template DNA was 4 µl of snail DNA diluted to 1:10. PCR conditions were as described by Caron *et al.*, (2011). All PCR products were visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5 % agarose gel. The expected size of the ITS-2 product was 401 bp for *G. truncatula* and 395 bp for *R. balthica* (Bargues *et al.*, 2001). As snails are known to contain inhibitors of PCRs, this PCR also served as an internal control when performing identification of *F. hepatica* infection (see 4.2.4) in snails, since its absence indicates inhibitory factors are present.

Given the similarity in the size of the ITS-2 PCR product a restriction enzyme digestion was used to differentiate between the *R. balthica* (395 bp) and *G. truncatula* (401 bp) products. An *in silico* restriction map was produced for *G. truncatula* and *R. balthica* ITS-2 sequences (AJ243017 and AJ319633, respectively; Bargues *et al.*, 2001) using NEBcutter (http://nc2.neb.com/NEBcutter2/). Criteria used to select restriction enzymes included: production of fragments of sufficient size to be visible on gel electrophoresis (minimum 100 bp), those enzymes that avoided areas of degeneracy between these sequences and the other sequences reported by Bargues *et al.*, (2001); and *R. balthica* and *G. truncatula* fragments that could readily differentiate between the two sequences. The restriction enzyme chosen was PstI,

which cut the 401 bp *G. truncatula* product, but does not cut the *R. balthica* product. The restriction enzyme reaction used 10 µl of PCR product, 1 µl of PstI (10 $U\mu l^{-1}$; Roche, Sussex, UK), 1.5 µl of 10 X SuRE/Cut Buffer H (Roche) and made up to 15 µl with ddH_2O. The plasmid pDEST17 (kindly provided by Mr Stuart Ainsworth, University of Liverpool) was used as a positive control. The restriction enzyme reactions were incubated for 1 hour at 37^oC and the entire reaction mixture was visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5 % agarose gel.

4.2.4 Identification of Fasciola hepatica *infection in snails by PCR*

A number of published methods have been reported for the detection of *F. hepatica* by PCR, including those that have been used to detect *F. hepatica* infection within snails (Table 4.1; see also Chapter 1). Each PCR was performed as cited in the literature (Table 4.1) with the following modifications (i) Biomix Red (Bioline) was used as the PCR reaction mix, and (ii) template DNA was 4 µl of 1 in 10 dilution of extracted snail DNA. Each PCR was evaluated for its ability to detect *F. hepatica* DNA in infected snails (see 4.2.4.1 and 4.2.4.2). PCRs were conducted with the following controls (i) DNA from *G. truncatula* known to be infected with *F. hepatica*, (ii) *G. truncatula* free from infection with *F. hepatica*, (iii) *F. hepatica* adult DNA, and (iv) negative control (ddH_2O). Controls (i) and (ii) were used at 1 in 100 dilution; control (iii) was used at 0.1 ng per reaction. All PCR products were visualised using SYBR Safe DNA stain (Life Technologies) on a 1.5 % agarose gel.

4.2.4.1 Sensitivity of the PCRs to detect Fasciola hepatica *infection in snails*

To evaluate their sensitivity, all PCRs were performed with template DNA from adult *F. hepatica* at serial 10 fold dilutions (10 ng, 1ng and 0.1 ng per reaction) and from 50, 30 and 10 metacercariae both fresh (one week old), and stored (6 months at 4^oC). DNA was extracted from the metacercariae as described in section 4.2.2 and 4 µl of the eluted DNA was used in each PCR reaction.

Table 4.1: Details of the PCRs used to detect *Fasciola hepatica* infection in snails

Target DNA sequence	Sequence of PCR primers (5'to 3')		Size of product (bp)	Reference citing primers	Reference citing PCR conditions	Our Reference
	Forward	Reverse				
ITS-2	FH-ITS2-SPEC-F: CTTATGATTTCTGGGATAATT	FH-ITS2-SPEC-R: CCGTCGCTATATGAAAA	112	Kralova-Hromadova *et al.*, (2008)	Novobilsky *et al.*, (2013)	Novobilsky ITS-2
ITS-2	ITS2F: GTGCCAGATCTATGGCGTTT	ITS2R: ACCGAGGTCAGGAAGACAGA	292	Robles-Perez *et al.*, (2013)		Robles-Perez ITS-2
Repetitive DNA: unknown location (Kaplan *et al.*, 1995)	Fsh1: GATCAATTCACCCATTTCCGTT AGTCCTAC	Fsh2: AAACTGGGCTTAAACGGCGTCC TACGGGCA	124*	Caron *et al.*, (2007)	Caron *et al.*, (2011)	Kaplan 124
RAPD of unknown location	FhepF: GCGGCCAATATGAGTCA	FhepR: CTGGAGATTCCGGTTACCAA	391	McGarry *et al.*, (2007)		McGarry RAPD
Mitochondrial DNA^	FHF: GTTTTTTAGTTGTTTGGGGTTTG	FHGR: ATAAGAACCGACCTGGCTCAC	1031	Le *et al.*, (2012)		Le mtDNA

bp = base pairs; * produces a ladder pattern due to tandem repeats; ^ targets the *COX1*, *transfer RNAs* for cysteine and threonine, and part of the 16S rRNA

4.2.4.2 Specificity of PCRs to detect Fasciola hepatica *infection in snails by amplification of ITS-2*

To assess the specificity of the two potential ITS-2 primer sets to identify *F. hepatica*, a sequence comparison of ITS-2 regions from related trematodes was performed. Searches conducted in February 2015 using GENBANK identified multiple sequences for *F. hepatica* (189 ITS-2 sequences), *Fasciola gigantica* (106 sequences) *Fascioloides magna* (7 sequences), *Dicrocelium dendriticum* (58 sequences), *Paragonimus westermani* (47 sequences) and *Paramphistomum cervi / Calicophoron daubneyi* (1 / 2 sequences).

To provide additional 5.8S, ITS-2 and 28S sequence data, products were amplified from adult *F. hepatica* DNA from a *F. hepatica* parasite from each of four sources (infected sheep in England, Wales and Scotland and an infected cow, Wales). The primers were as described by Itagaki and Tsutsumi, (1998) based on their ability to amplify this region from *Fasciola* spp. and other trematodes (Rinaldi *et al.*, 2005; Sanabria *et al.*, 2011): forward primer ITS-2 F (5' – TGT GTC GAT GAA GAG CGC AG – 3') and reverse primer ITS-2 R (5' – TGG TTA GTT TCT TTT CCT CCG C – 3'). The PCR reaction was as described by Itagaki and Tsutsumi, (1998) with the following modifications (i) Biomix Red (Bioline) was used as the PCR reaction mix, and (ii) 20 ng of template DNA was used. PCR conditions were as described by Rinaldi *et al.*, (2005). PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced using the ITS-2 F and ITS-2 R primers by Source BioScience (Nottingham, UK). Sequences were analysed using the Staden Package (http://staden.sourceforge.net/). Pregap4 was used for shotgun assembly using the default settings and sequences were viewed in Gap4 and manually checked. Alignment of all sequences was performed using the Clustal W multiple alignment with BioEdit 7. Consensus sequences were created and the primers for Robles-Perez ITS-2 and Novobilsky ITS-2 were mapped.

4.2.5 Maintenance of snails under laboratory conditions

I am grateful to Miss Katherine Allen, Miss Alice Balard, Mrs Cathy Glover and Mrs Catherine Hartley for the collection of soil and production of blue-green algae (*Oscillatoria* sp.), used in the maintenance of snails under laboratory conditions at the University of Liverpool. Snails were routinely kept on mud pans made with clay soil and fed with blue-green algae grown on compressed mud which

had been dampened with algal fertiliser. The tray of algae was maintained under mercury vapour strip lighting approximately 80 cm above the tray and at a temperature of 24°C.

4.2.6 Experimental infection of laboratory maintained Galba truncatula *with* Fasciola hepatica

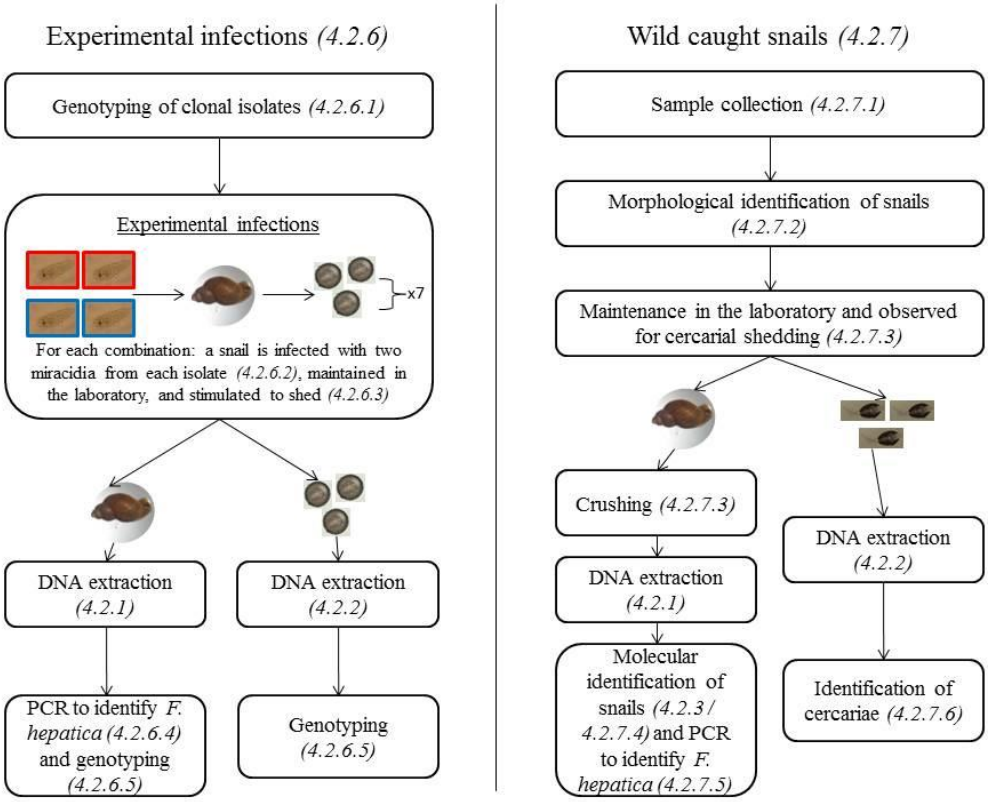

Figure 4.1: Schematic to show the experimental design for (i) experimental infections of laboratory maintained *Galba truncatula* with *Fasciola hepatica* and (ii) sampling wild caught snails and assessment for infection with *F. hepatica*; section references are shown in italics

4.2.6.1 Genotyping of clonal isolates of Fasciola hepatica

As part of a BBSRC grant (BB1002480/1) six clonal isolates of *F. hepatica* were derived. All isolates were originally isolated from natural infections of *F. hepatica.* A MLG was produced for each of the six isolates as described in section 3.2.4. Each isolate had a unique MLG making it possible to differentiate between them, with four isolates (B, C, N and T) showing the greatest levels of differentiation at each locus. Four loci that were particularly useful for differentiating all four isolates were Fh_1, Fh_4, Fh_6 and Fh_11.

4.2.6.2 Infection of Galba truncatula *with* Fasciola hepatica *miracidia*

Eggs from each of the chosen isolates were incubated with aerated tap water (pH 7) in petri dishes covered with foil for a minimum of two weeks at 25^{o}C to allow for development of the miracidia within the egg (Boray, 1969). Seven combinations of the isolates were used to infect snails: TN, BC, TB, NB, TC, NC, and TNBC. Two miracidia from each isolate were used for the infection, each combination was replicated a total of seven times. All infections within one combination were performed on the same day, but the seven combinations of infections were produced over a two week period.

Infections were performed in 12-well plates. Snails were "shocked" by placing in a fridge at 4^{o}C for 30 minutes prior to infection (Abrous *et al.*, 2001a). Eggs were removed from the incubator, and exposed to light to stimulate hatching of the miracidia. Miracidia were isolated under a dissection microscope (5X magnification) and snails were exposed to the miracidia. Snails were visualised under the dissection microscope until the miracidia had entered the snail, and were then fed algae and left overnight before being transferred for maintenance.

4.2.6.3 Maintenance of experimentally infected snails and shedding of cercariae by infected snails

Snails which had been infected with miracidia of the same origin were kept together on the same mud pan until the point of shed, at which point any snails that shed were kept individually. Snails were kept on mud in 6-well plates (maximum of 4 snails per well). Snails and their containers were washed every 2 to 3 days using aerated tap water and fed every 1 to 2 days with algae. Snails were stimulated to shed from six to eight weeks post infection at two weekly intervals on five occasions (up to a maximum of 16 weeks post infection). At the end of this period surviving snails were killed and, along with any snails that died during the experiment but had survived to 30 days post exposure, DNA was extracted (see 4.2.1), either immediately or after storage of the snail at -80^{o}C.

The optimal conditions under which *F. hepatica*-infected snails shed cercariae was developed previously by Miss Katherine Allen and involved feeding snails 24 hours prior to shedding with approximately double the amount of blue-green algae. Snails were placed in strips of visking tubing (Membra-Cel MC18, Sigma-Aldrich) with aerated tap water and kept separate from one another using clip-

locks. The entire apparatus was placed into a beaker of tap water and cooled down to 7 ± 1^{o}C using ice. The temperature was maintained for 1 hour and the beaker was then exposed to mercury vapour strip lighting approximately 80 cm above the laboratory bench and a temperature of 24^{o}C. Snails were left overnight and visking tubing was observed under a dissection microscope the following morning at up to 5X magnification to check for metacercariae. Metacercariae encysted on the visking tubing were removed by scraping, and DNA was extracted from metacercariae at each shedding time-point separately and was performed immediately as described in section 4.2.2.

4.2.6.4 PCRs to identify those snails that had been infected with Fasciola hepatica

All snails from which DNA was extracted were subjected to Novobilsky ITS-2 and Kaplan 124 PCRs (see 4.2.4 and Table 4.1) to detect infection with *F. hepatica*. An internal positive control was performed using the Bargues ITS-2 PCR (see 4.2.3).

4.2.6.5 Genotyping to determine the MLG of (i) snails showing evidence of Fasciola hepatica *infection by PCR, and (ii) metacercariae shed from infected snails*

All snails and metacercariae from which DNA was extracted were subjected to genotyping as described in section 3.2.4. The following modifications were required: (i) 4 µl of template DNA was used, (ii) to combat inhibitory factors, DNA extracted from snails was first diluted 1 in 10 with TE; and (iii) the number of PCR cycles was increased to 37 for multiplex and 40 for singleplex (locus Fh_1 only).

4.2.7 Sampling wild caught snails and assessment for Fasciola hepatica *infection (Figure 4.1)*

4.2.7.1 Sample Collection

Snails were collected from farms with known *F. hepatica* infection in their livestock. As part of a grant to improve the control of liver fluke infection in cattle in the UK (BB/K015591/1) the Centre for Ecology and Hydrology collected snails from a farm in Scotland and sent them to the University of Liverpool. With the assistance of Mr Daniel Smith (University of Liverpool) snails were collected from two sheep farms in North Wales. Habitats typical of *G. truncatula* were identified but all snails

found were collected and taken to the University of Liverpool. A total of 162 snails were collected, details of which are shown in Table 4.2.

Table 4.2: Details of farms, sampling dates and species of snails collected

Farm (Location)	**Collection Date**	**Morphological species identification (no. of snails collected)**	**No. of snails shed once (%)**	**No. of snails shed twice (%)**	**No. of snails shed three times (%)**	**Subset of snails subjected to PCR**
1 (Scotland)	September 2014	*Radix balthica* (7)	7 (100)	5 (71.4)	5 (71.4)	7
1 (Scotland)	October 2014	*R. balthica* (45)	45 (100)	44 (97.8)	44 (97.8)	13
2 (North Wales)	October 2014	*Potamopyrgus jenkinsi* (22)	22 (100)	20 (90.9)	20 (90.9)	21
		Succinea spp. (4)	4 (100)	4 (100)	3 (75)	4
3 (North Wales)	October 2014	*Succinea* spp. (21)	20 (95.2)	19 (90.5)	19 (90.5)	21
3 (North Wales)	November 2014	*Galba truncatula* (52)	46 (88.5)	37 (71.2)	0 (0)	52
		P. jenkinsi (10)	10 (100)	10 (100)	10 (100)	10
		Succinea spp. (1)	1 (100)	1 (100)	0 (0)	1
Total no. of snails (%)		162 (100)	155 (95.7)	140 (86.4)	101 (62.3)	129 (79.6)

4.2.7.2 Morphological identification of wild caught snails

On arrival at the University of Liverpool, snails were morphologically identified under a dissection microscope at up to 5X magnification. A dichotomous key was used, which allowed morphological identification of the snails to the genus level in all cases, and the species level in the majority of cases (Macan, 1977).

Snails at Farm 1 were collected from a stream; they were identified as *R. balthica* by the following characteristics: absence of an operculum, a coil shell (nearly in one plane) with a short pointed spire that was shorter than the aperture, the shell was not transparent, and the last whorl was less expanded meeting the body at less than a right angle (Figure 4.2A).

Snails at Farm 2 were collected from a stream adjacent to a field where sheep, identified with *F. hepatica* infection, were known to have grazed. The snails were identified as either *P. jenkinsi* by the following characteristics: presence of an operculum, a thick shell with 5.5 whorls the last of which is large (Figure 4.2B); or *Succinea* spp. (Figure 4.2C; identified to the genus level only) by the following characteristics: no operculum, a spiral shell, and eyes at the tip of a second pair of tentacles. The shells were yellow and the snail did not fit into its shell.

Snails at Farm 3 were collected from a rainwater draining furrow on an area of the field that sloped down to a ditch, there were numerous collections of water with decaying vegetation and algal blooms. The *P. jenkinsi* and *Succinea* spp. were identified as above; *G. truncatula* were identified by the following characteristics: lack of an operculum, a right-handed spiral shell with a tall spire nearly as long, or longer, than the aperture; adults were between 8 and 12 mm high with an aperture about half the height of the shell and 4.5 whorls (Figure 4.2D).

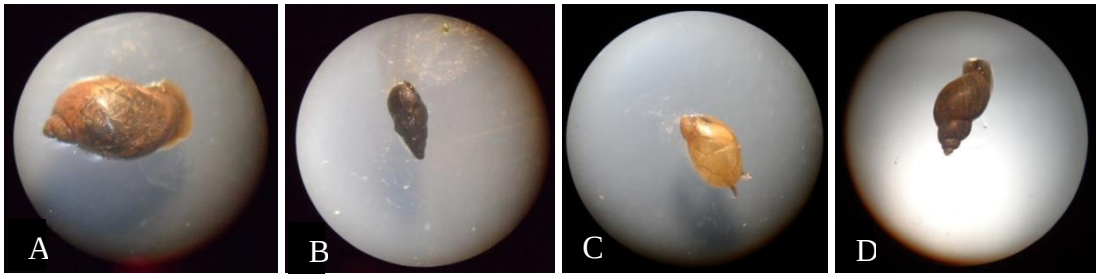

Figure 4.2: Wild caught snails; **A**: *Radix balthica*; **B**: *Potamopyrgus jenkinsi*; **C**: *Succinea* spp.; **D**: *Galba truncatula*

4.2.7.3 Maintenance of wild caught snails from the field and shedding of cercariae by infected snails

All snails were separated into batches by farm and species, and maintained in the laboratory in conditions similar to where they were found: if found in soil, in glass (diameter 140 mm, depth 75 mm) or plastic containers (length 165, width 115, depth 40 mm) on clay soil; if found in water, in plastic containers (length 165, width 115, depth 40 mm) with a small volume of aerated tap water. Snails were maintained in the laboratory for up to 61 days: the snails and their containers were washed every 2 to 3 days using aerated tap water and fed every 1 to 2 days with either blue-green algae, baby gem lettuce or fish food (Aquarin® Goldfish Food). Snails were kept at a constant temperature of 24^{o}C.

All snails were subject to a shedding protocol optimised for *G. truncatula* (see 4.2.6.3) except that snails were covered with aerated tap water in individual wells of 12- or 24- well plates, and cooled by storing at 4^{o}C for two hours. Shed cercariae and metacercariae were visualised under a dissection microscope at up to 5X magnification. Cercariae were collected and, depending on the number of cercariae, were stored in 50 to 150 µl of distilled water at -80^{o}C before DNA extraction (see 4.2.2). Any snails that shed were then kept in individual wells of a 6-well plate. Snails were initially kept for two weeks post-collection and then exposed to conditions to stimulate shedding up to three times at two weekly intervals. Snails that died during this period, and those still alive at the end of this period, were crushed using a scalpel and visualised under a dissection microscope at up to 5X magnification to check for evidence (visualisation of rediae or cercariae) of *F. hepatica* infection. DNA was then extracted (see 4.2.1) from all snails either immediately or following storage at -80^{o}C.

4.2.7.4 Molecular identification of wild caught snails

Confirmation of morphological identification was performed for all snails by amplification of an ITS-2 region using Bargues ITS-2 (see 4.2.3). One or two ITS-2 amplicons, considered to be representative of each species that had been morphologically identified, were purified using a QIAquick PCR purification kit (Qiagen); and sequenced using the LT-1 and ITS-2 RIXO primers by Source BioScience. Sequences were analysed using the Staden Package as described in 4.2.4.2. Consensus sequences were run through NCBI Nucleotide BLAST

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find highly similar sequences (searches performed June 2015).

4.2.7.5 PCRs to identify those snails that had been infected with Fasciola hepatica

All snails from which DNA was extracted were evaluated for infection with *F. hepatica* using Novobilsky ITS-2, Kaplan 124, McGarry RAPD, and Le mtDNA PCRs (see 4.2.4 and Table 4.1). An internal positive control was performed using the Bargues ITS-2 PCR (see 4.2.3).

4.2.7.6 Identification of cercariae shed from infected wild caught snails

Two snails collected from the field shed cercariae. These cercariae did not encyst and did not resemble cercariae of *F. hepatica* morphologically. To identify these cercariae, DNA was extracted (see 4.2.2), and the ITS-2 region was amplified using primers designed by Itagaki and Tsutsumi (1998) and as described in section 4.2.4.2. PCR products were purified using QIAquick PCR purification kit and sequenced using the ITS-2 F and ITS-2 R primers (Itagaki and Tsutsumi, 1998) by Source BioScience. Sequences were analysed using the Staden Package as described in 4.2.4.2. Consensus sequences were run through NCBI Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find highly similar sequences and somewhat similar sequences (searches performed February 2015).

To further evaluate the specificity of the PCRs to detect *F. hepatica* (see 4.2.4.2), these cercariae were also subject to the PCRs described in 4.2.4 and Table 4.1 with the only modification being to use neat, rather than diluted, template DNA.

4.2.8 Ethical approval

Ethical approval was received from the University of Liverpool's Veterinary Research Ethics Committee (VREC142a).

4.3 RESULTS

4.3.1 Can a snail be experimentally infected and subsequently shed Fasciola hepatica *of more than one genotype?*

4.3.1.1 Multilocus genotyping of four clonal isolates of Fasciola hepatica

The genotypes of the four clonal isolates used for the experimental infections are shown in Table 4.3. The MLG for each isolate is distinct and a number of alleles are unique to an isolate, in particular loci Fh_1 and Fh_11 are represented by a different genotype for each isolate.

Table 4.3: Multilocus genotypes of the B, C, N and T clonal isolates that were used for experimental infections

Locus	**Genotype* of each clonal isolate**			
	B	**C**	**N**	**T**
Fh_1	0808	1111	1016	0606
Fh_2	0845	1419	1415	0808
Fh_3	0808	0708	0708	0708
Fh_4	1618	1620	1619	1420
Fh_5	2424	2732	2730	2731
Fh_6	1433	3032	1629	1416
Fh_7	1111	1112	1313	1212
Fh_8	1116	1212	1212	1119
Fh_9	0607	0707	0707	0607
Fh_10	0915	0911	1114	0611
Fh_11	0714	1112	1313	0815
Fh_12	1015	1516	0510	1010
Fh_13	0811	0808	0811	0816
Fh_14	1718	1818	0808	1719
Fh_15	0914	0914	0914	1414

***** alleles are identified by the number of repeats (rather than the size of the fragment) and are in a two-figure format with genotypes in a four-figure format made up of two alleles

4.3.1.2 Snail survival and detection of Fasciola hepatica *infection by shedding of cercariae*

Of the 49 snails infected (7 snails per combination of isolates) a total of 43 snails (87.8 %) survived to at least 30 dpi (Table 4.4). Snails were subjected to shedding five times and across the entire experiment 25 snails survived (51 %; 5th shed: 112 ± 2 dpi). Snails infected with some combinations of *F. hepatica* miracidia survived for a longer time frame than others: TB, NB, NC and TNBC all had > 70 % survival until the end of the experiment at 112 ± 2 dpi (Table 4.4).

Table 4.4: Results of the experimental infection of *Galba truncatula* with *Fasciola hepatica* miracidia of known genotypes in relation to survival

Combination of *F. hepatica* miracidia used for infection	No. of snails surviving at each time-point (%)					
	30 dpi	1st Shed: 42* / 56 ± 2 dpi	2nd Shed: 70 ± 2 dpi	3rd Shed: 84 ± 2 dpi	4th Shed: 98 ± 2 dpi	5th Shed: 112 ± 2 dpi
TN	4 (57.1)	3 (42.9)	0 (0)	0 (0)	0 (0)	0 (0)
BC	6 (85.7)	4 (57.1)	2 (28.6)	2 (28.6)	1 (14.3)	1 (14.3)
TB	7 (100)	6 (85.7)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)
NB	6 (85.7)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)
TC	7 (100)	7 (100)	6 (85.7)	4 (57.1)	4 (57.1)	3 (42.9)
NC	7 (100)	6 (85.7)	6 (85.7)	6 (85.7)	6 (85.7)	6 (85.7)
TNBC	6 (85.7)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)	5 (71.4)
All combinations	43 (87.8)	36 (73.5)	29 (59.2)	27 (55.1)	26 (53.1)	25 (51.0)

*The TN combination were first shed at 6 weeks post infection, all other combinations were shed at 8 weeks post infection; dpi = days post infection

All snails that survived to 30 dpi (n = 43) were subjected to the conditions that stimulate shedding of cercariae. Only two snails (4.1% of all snails infected) shed cercariae, both of which were infected with the NB isolate. One snail first shed at time-point 2 (69 dpi) and at each of the subsequent time points: 3rd Shed (83 dpi), 4th shed (97 dpi) and 5th shed (111 dpi); the number of metacercariae produced at each shed was 89, 88, 139 and 136, respectively (mean = 113 ± SD 28.3). The

second snail first shed at time-point 3 (83 dpi) and again at the 4th (97 dpi) and 5th (111 dpi) shed, and the number of metacercariae produced at each shed was 85, 76 and 172, respectively (mean = 114.3 ± SD 58.8).

4.3.1.3 Detection of Fasciola hepatica *infection in experimentally infected snails by PCR*

DNA was extracted from all snails that survived to 30 dpi (n = 43). As an internal positive control the Bargues ITS-2 PCR was carried out for all 43 snails and amplified a product from 28 snails (65.1 %). Detection of *F. hepatica* DNA within the snail was determined by two PCRs: (i) Novobilsky ITS-2 and (ii) Kaplan 124. *Fasciola hepatica* DNA was amplified from 20 snails (46.5 %) by the Novobilsky ITS-2 PCR and all but one of these (lane 38; Figure 4.3) was recorded as positive by the Kaplan 124 PCR (Figure 4.3). All snails positive for *F. hepatica* DNA were genotyped to produce a MLG.

4.3.1.4 Multilocus genotyping of Fasciola hepatica *DNA from experimentally infected snails*

Microsatellite genotyping provided robust alleles for five snails (25 %; snail 10, 24, 25, 29 and 31; Table 4.5). Snail 10 was infected with isolates B and C; the majority of loci genotyped match to isolate B. However locus Fh_4 and Fh_10 showed alleles only found in isolate C. Therefore there could be a low level of DNA from isolate C also present. Snails 24 and 25 were both infected with isolates N and B, the majority of loci showed a match to isolate B, with some alleles being either N or B; no alleles uniquely identified isolate N as present in the infection. Snails 29 and 31 were infected with isolate T and C and the genotyping supports the presence of a mixed infection. At most loci all alleles expected from a mixed infection were identified except at locus Fh_1 (snail 29 only), Fh_2, Fh_4 (snail 29 only), Fh_5 (snail 29 only), Fh_6 (snail 31 only), Fh_9 (snail 31 only) and Fh_11.

4.3.1.5 Multilocus genotyping of Fasciola hepatica *DNA from metacercariae*

The two snails that shed cercariae were infected with both the N and B isolate. The results of microsatellite genotyping of the metacercariae from each shed are shown in Table 4.6. The MLG at each time-point is consistent with the alleles known to be present in only one isolate, isolate B. This result is consistent with the

results of genotyping the *F. hepatica* infection within the snail where both snail 24 and 25 showed no evidence of alleles unique to isolate N (Table 4.5).

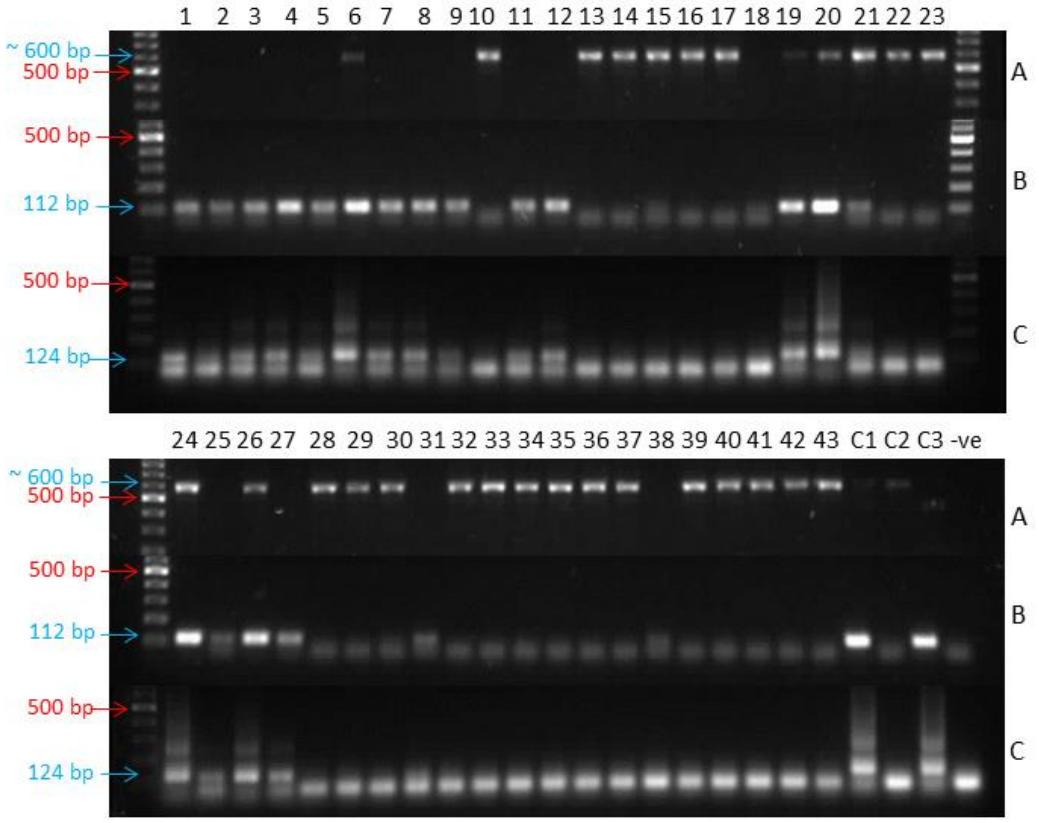

Figure 4.3: Composite gels showing results of PCRs to identify *Fasciola hepatica* infection within the experimental snails; **A**: Snail internal positive control Bargues ITS-2 (~ 600 bp due to microsatellite repeat region); **B**: Detection of *F. hepatica by* Novobilsky ITS-2 (112 bp); **C**: Detection of *F. hepatica* by Kaplan 124 (124 bp). Lanes 1 to 43: snails 4 to 7, 9 to 21, 23 to 42 and 44 to 49; C1: *G. truncatula* known to be infected with *F. hepatica*; C2: *G. truncatula* free from infection with *F. hepatica*: C3: *F. hepatica* adult DNA; -ve: negative control (ddH_2O)

Table 4.5: Results of the multilocus genotyping of *Fasciola hepatica* infection using DNA extracted from experimentally infected snails

Locus	Alleles* detected for each snail successfully genotyped				
	Snail 10	**Snail 24**	**Snail 25**	**Snail 29**	**Snail 31**
Fh_1	08	08	08	11	06, 11
Fh_2	45	08, 45	08	08, 19	08, 19
Fh_3	08	08	08	07, 08	07, 08
Fh_4	16, 20	16	16	14, 20	14, 19, 20
Fh_5	24	24	24	27, 32	27, 31, 32
Fh_6	33	22, 41	22	14, 16, 30, 32	14, 16, 30
Fh_7	11	11	11	11, 12	11, 12
Fh_8	11, 16	11, 16	11, 16	11, 12, 19	11, 12, 19
Fh_9	06, 07	06, 07	06, 07	06, 07	07
Fh_10	09, 11	09	15	06, 09, 11	06, 09, 11
Fh_11	07, 14	07, 14	07, 14	08, 11, 15	08, 11, 12
Fh_12	10	10	10, 15	10, 15, 16	10, 15, 16
Fh_13	08, 11	08, 11	11	08, 16	08, 16
Fh_14	17	17	17, 18	17, 18, 19	17, 18, 19
Fh_15	09, 14	09, 14	09	09, 14	09, 14
Isolates infected	B and C	N and B	N and B	T and C	T and C
Isolates identified	B	B	B	T and C	T and C

* alleles are identified by a two figure code indicating the number of repeats

Table 4.6: Results of the multilocus genotyping of *Fasciola hepatica* DNA from metacercariae collected from experimentally infected snails

Locus	Alleles* detected for each group of metacercariae successfully genotyped						
	Snail 24				Snail 25		
	2nd shed	3rd shed	4th shed	5th shed	3rd shed	4th shed	5th shed
Fh_1	08	08	08	08	08	08	08
Fh_2	08, 45	08, 45	08, 45	08, 45	08	08	08
Fh_3	08	08	08	08	08	08	08
Fh_4	16	16	16	16	16	16	16
Fh_5	24	24	24	24	24	24	24
Fh_6	14, 33	14, 33	14, 33	14, 33	14	14	14
Fh_7	11	11	11	11	11	11	11
Fh_8	11, 16	11, 16	11, 16	11, 16	11, 16	11, 16	11, 16
Fh_9	06, 07	06, 07	06, 07	06, 07	06, 07	06, 07	06, 07
Fh_10	09, 15	09, 15	09, 15	09, 15	15	15	15
Fh_11	07, 14	07, 14	07, 14	07, 14	07, 14	07, 14	07, 14
Fh_12	10	10	10	10	10, 15	10, 15	10, 15
Fh_13	08, 11	08, 11	08, 11	08, 11	11	11	11
Fh_14	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18
Fh_15	09, 14	09, 14	09, 14	09, 14	09	09	09
Isolate identified	B	B	B	B	B	B	B

* alleles are identified by a two figure code indicating the number of repeats

4.3.2 Population structure of Fasciola hepatica *infecting wild caught snails in the field*

4.3.2.1 Detection of Fasciola hepatica *infection in wild caught snails from the field by observation of shedding of cercariae*

Of the snails which we tested for their capacity to shed cercariae (i.e. capable of contributing to transmission of *F. hepatica*) only two (3.85 %) of the 52 *G. truncatula* snails shed cercariae. This represented 1.23 % of all snails collected. One snail shed approximately 30 cercariae and the second snail shed < 10 cercariae. These cercariae failed to encyst and morphologically did not resemble *F. hepatica* (see 4.3.2.5). When crushed the two *G. truncatula* that shed cercariae showed no evidence of free cercariae and no obvious redial structures. All other snails were also crushed to provide a second check for *F. hepatica* infection, no snails showed evidence of rediae / cercariae.

4.3.2.2 Molecular identification of snails

From Farm 1 all 20 (100 %) *R. balthica* samples gave a product of the expect size (between 400 and 500 bp), two of which were sequenced and showed 100 % identity. A consensus sequence of 459 bp was generated. The top BLAST hit for this consensus showed 99 % identity across 88 % of the sequence (E-value = 0.0) and was confirmed as *R. balthica* (Accession numbers: HE573083.1 and HE573078.1). From Farms 2 and 3, only 3 (75 %) and 8 (38.1 %) of the *Succinea* spp. respectively gave a product of ~ 600 bp, one of which was sequenced to provide a 597 bp sequence. The top BLAST hit showed 100 % identity over 73 % of the sequence (E-value = 0.0) to *Succinea putris* (Accession number: KF887038). The *S. putris* sequence in GENBANK represents only partial sequence from the ITS-2, and likely explains the low query cover. From Farms 2 and 3, 21 (100 %) and 10 (100 %) of the *P. jenkinsi*, respectively, gave a product approximately 300 bp; one of which was sequenced to provide a sequence of 169 bp in length. The top BLAST hits showed 100 % identity across 100 % of the sequence (E-value = 8×10^{-82}) with multiple *Potamopyrgus antipodarum* (synonymous with *P. jenkinsi*) nucleotide sequences in the database (e.g., Accession Number: JF960509.1). From Farm 3, 29 (55.7 %) of *G. truncatula* samples gave a product between 400 and 500 bp (two of these were sequenced), and one (1.9 %) *G. truncatula* sample produced a product ~ 600 bp. The

two *G. truncatula* sequences showed 100 % identity to each other and gave a sequence of 464 bp. The top BLAST hit showed 100 % identity across 97 % of the sequence (E-value = 0.0) to *G. truncatula* (Accession Number: KC248372.1). The morphological identifications of all the field snails were therefore confirmed, along with identification to the species level for *S. putris*.

In order to differentiate between *R. balthica and G. truncatula* by size of the ITS-2 it was necessary to perform a restriction digest using enzyme PstI. The restriction enzyme cut the ITS-2 region of *G. truncatula* to produce two products but did not cut *R. balthica* (Figure 4.4). These findings were confirmed for all *R. balthica* and *G. truncatula* products generated with the Bargues ITS-2 PCR.

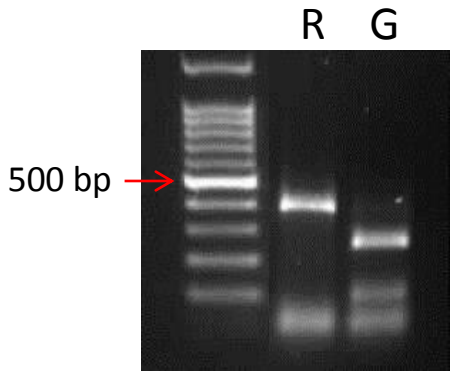

Figure 4.4: Gel following restriction digest of *Radix balthica* and *Galba truncatula* using PstI; **R**: restriction digest of *R. balthica* (395 bp); **G**: restriction digest of *G. truncatula* (99, 103, 298, 302 bp)

4.3.2.3 Sensitivity and specificity of the PCRs to detect Fasciola hepatica *infection in snails*

The sensitivity of five PCRs to detect *F. hepatica* infection in snails (Table 4.1) was evaluated. The Robles-Perez ITS-2, Novobilsky ITS-2 and Kaplan 124 PCRs were the most sensitive; the Le mtDNA was the least sensitive (Figure 4.5).

The specificity of the two PCRs that amplify regions of the ITS-2 (Robles-Perez ITS-2 and Novobilsky ITS-2) was evaluated by sequence comparison. The ITS-2 region of *F. hepatica* sequenced from Great Britain in this study showed 100 % identity with each other, and three nucleotide differences with *F. hepatica* ITS-2 sequences obtained from GENBANK. There were seven nucleotide differences in the *F. gigantica* ITS-2 consensus; three nucleotide differences in the *D. dendriticum* ITS-2 consensus; no differences in the *F. magna* ITS-2 consensus; two differences in

the *C. daubneyi* ITS-2 consensus (which showed nine nucleotide differences to *Pm. cervi*), and 16 nucleotide differences in the *Pg. westermani* consensus. Overall the ITS-2 shows high intraspecific conservation. When compared to *F. hepatica* interspecific identity was 96.1 % for *F. gigantica* and 92.6 % for *F. magna*. The remaining trematodes showed < 70 % identity to *F. hepatica*. The Robles-Perez ITS-2 primers are in areas of the ITS-2 that are conserved amongst all the trematodes studied, whilst in comparison the Novobilsky ITS-2 primers show greater specificity to *F. hepatica* (Figure 4.6).

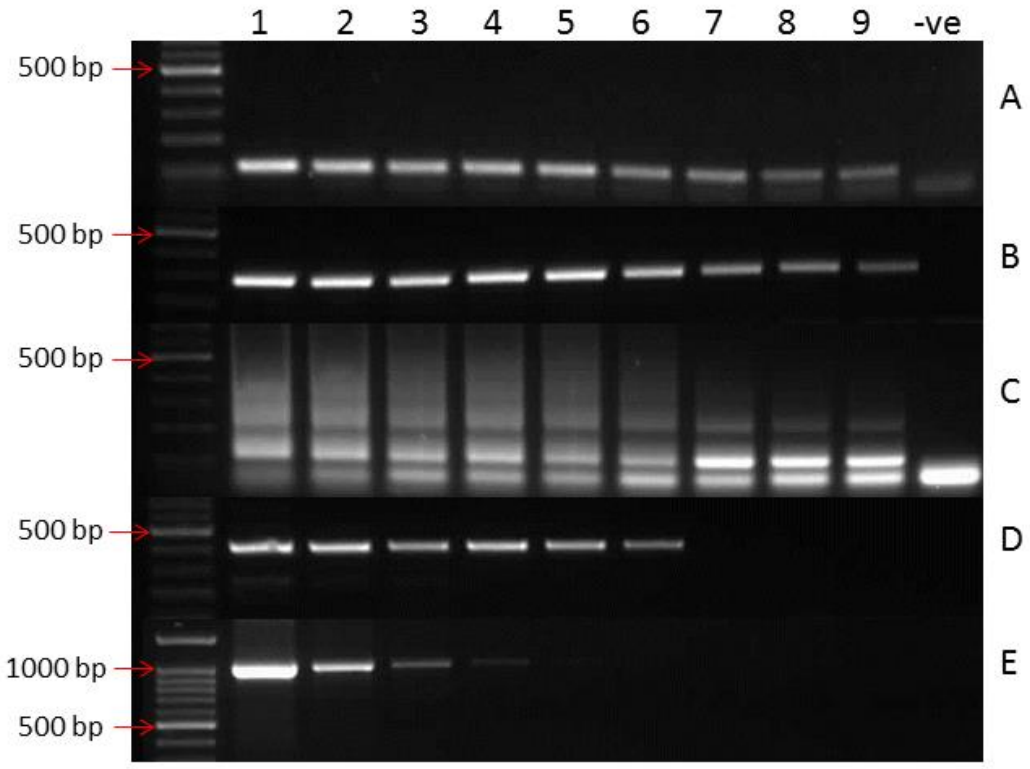

Figure 4.5: Sensitivity of the PCRs to detect *Fasciola hepatica*; **A**: Novobilsky ITS-2 (112 bp); **B**: Robles-Perez ITS-2 (292 bp); **C**: Kaplan 124 (124 bp); **D**: McGarry RAPD (391 bp); **E**: Le mtDNA (1031 bp; Table 4.1). Lanes 1 to 3: *F. hepatica* adult DNA 10 fold serial dilutions 10ng, 1ng and 0.1ng; Lanes 4 to 6: 50, 30 or 10 one week old metacercariae; Lanes 7 to 9: 50, 30 or 10 six month old metacercariae; –ve: negative control (ddH_2O)

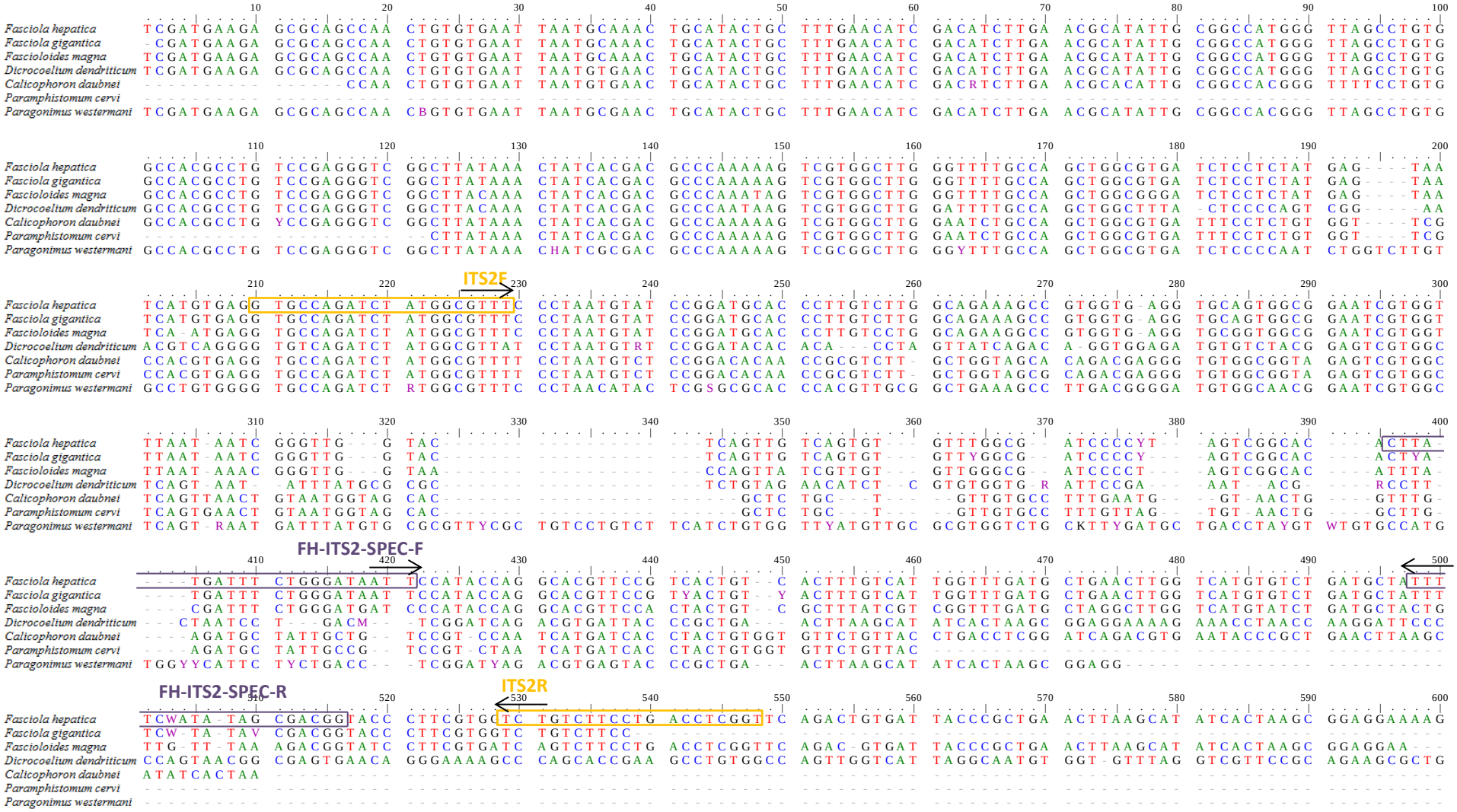

Figure 4.6: Alignment of consensus sequences of the ITS-2 region of *Fasciola hepatica*, *Fasciola gigantica*, *Fascioloides magna*, *Paramphistomum cervi*, *Calicophoron daubnei*, *Dicrocelium dendriticum* and *Paragonimus westermani*; Primers are mapped from Robles-Perez ITS-2 (ITS2F and ITS2R) and Novobilsky ITS-2 (FH-ITS2-SPEC-F and FH-ITS2-SPEC-R; Table 4.1) PCRs

4.3.2.4 Molecular identification of Fasciola hepatica *infection within snails*

Due to the possibility of PCR inhibition a snail negative for *F. hepatica* infection was defined as a snail for which the snail-specific Bargues ITS-2 PCR was successful but for which a product was not generated for any of the *F. hepatica* PCRs: Novobilsky ITS-2, Kaplan 124, McGarry RAPD and Le mtDNA.

The Novobilsky ITS-2 PCR generated products of the expected size for one *R. balthica*, four *S.* putris (one from Farm 2 and three from Farm 3), one *P. jenkinsi* (from Farm 2) but none of the *G. truncatula*. However, no *F. hepatica* DNA was detected in any of these six samples using the Kaplan 124, McGarry RAPD or Le mtDNA PCRs.

4.3.2.5 Molecular identification of cercariae shed from Galba truncatula *collected from Farm 3*

The PCR products amplified from cercariae shed from the two *G. truncatula* snails from Farm 3 were sequenced and showed 100 % identity to each other across the 454 bp ITS-2 region. Based on BLAST analysis, conducted as described in section 4.2.7.6, plus awareness of the countries these intermediate hosts, definitive hosts and Digenea are found, the two best candidates were *Plagiorchis maculosus* which had 87 % identity over 94 % of the sequence (E-value: 3×10^{-125}; Accession Number: KJ533390.1) and *Dolichosaccus symmetrus* which had 92 % identity over 65 % of the sequence (E-value: 4×10^{-109}; Accession Number: L01631.1).

The consensus sequence from the unidentified cercariae shed here were aligned with *Plagiorchis* spp. (12 sequences) and *Dolichosaccus* spp. (2 sequences) ITS-2 sequences from GENBANK. The alignment showed greater identity to the *Plagiorchis* spp. with 82.3 % identity across the aligned portion (Figure 4.7), whereas there was only 44.3 % identity with *Dolichosaccus*.

To further assess the specificity of the PCRs to detect *F. hepatica*, the DNA extracted from these unidentified cercariae were subjected to the PCRs detailed in Table 4.1. The results of these PCRs are shown in Figure 4.8. Products of the expected size were generated for the Novobilsky ITS-2 and Robles-Perez ITS-2 PCRs; products of different sizes to what was expected were generated for the Kaplan 124 and the McGarry RAPD PCRs, and no products were generated from the Le mtDNA PCR.

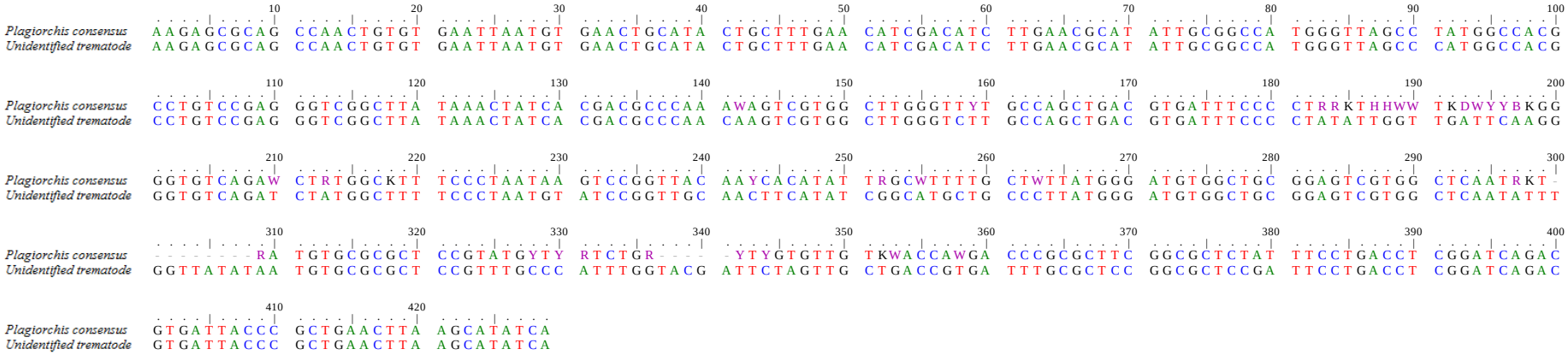

Figure 4.7: Alignment of the ITS-2 of *Plagiorchis* spp. (consensus sequence created from 12 sequences deposited in GENBANK) and ITS-2 of the unidentified trematode cercariae shed from two of the wild caught snails

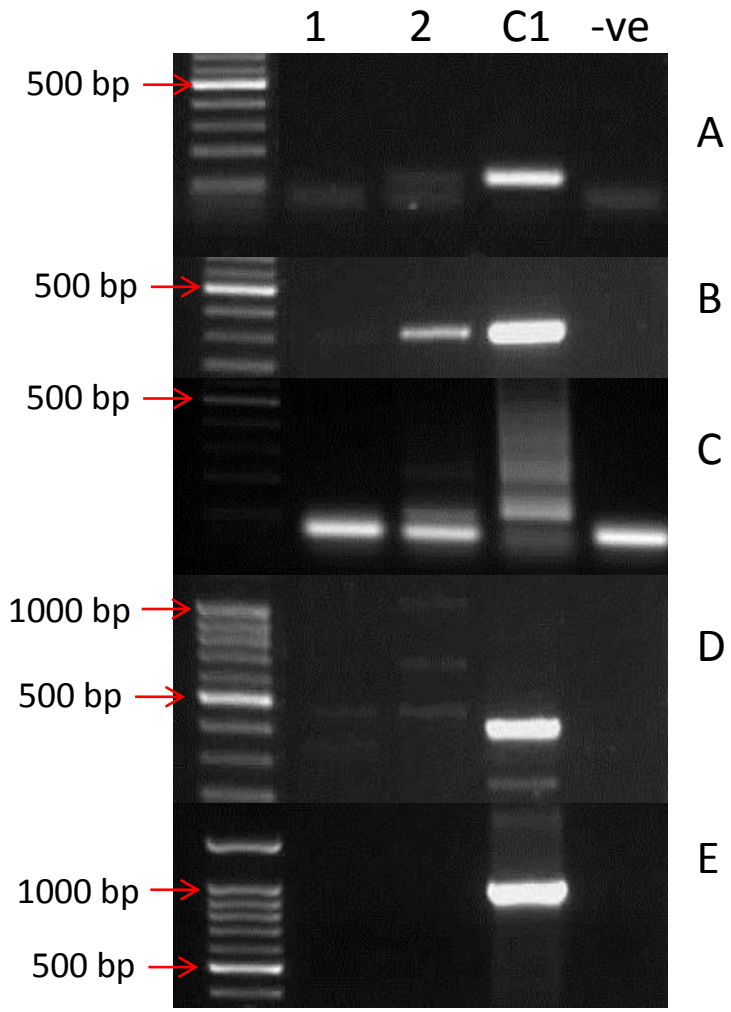

Figure 4.8: Further assessment of the specificity of PCRs that identify *Fasciola hepatica*, by using DNA from unidentified cercariae; **A**: Novobilsky ITS-2 (expected product size for *F. hepatica* 112 bp); **B**: Robles-Perez ITS-2 (expected product size for *F. hepatica* 292 bp); **C**: Kaplan 124 (expected product size for *F. hepatica* 124 bp); **D**: McGarry RAPD (expected product size for *F. hepatica* 391 bp); **E**: Le mtDNA (expected product size for *F. hepatica* 1031 bp); 1: < 10 unidentified cercariae; 2: 30 unidentified cercariae; C1: *F. hepatica* adult DNA; -ve: negative control (ddH_2O)

4.4 DISCUSSION

4.4.1 Snails can be infected with Fasciola hepatica *miracidia of more than one genotype*

We have shown that *G. truncatula* can be infected with two *F. hepatica* isolates each with a distinct genotype. We were not able to show that these snails are involved in transmission of the two isolates as neither of these snails shed cercariae. However, this is probably due to a low rate of shedding throughout the experiment. The 30 day post exposure survival rate (87.8 %) in this study is similar to that reported by others (e.g., Rondelaud *et al.*, 2014a). However, only 4.7 % of these snails went on to shed cercariae and this is low compared to other studies which reported > 20 % of infected snails shed cercariae, and in some cases > 60 or 70 % of snails (Vignoles *et al.*, 2006; 2011; Sanabria *et al*., 2012; Rondelaud *et al.*, 2014b). It has been suggested that if a snail has adequate nutrition then it is likely to be able to support infection by more than one miracidia, and multiple sporocysts have been identified within snails (Rondelaud *et al.*, 2004). We have shown that miracidia of distinct genotypes can infect a snail. If this translates through to transmission then it may help to explain the level of genetic variation seen in the definitive host.

Whilst neither of the snails that showed evidence of infection with more than one isolate shed cercariae, cercariae were shed by two snails infected with parasites of a single genotype. The metacercariae collected from successive sheds were analysed separately: no difference in the genotype was seen, suggesting that shedding is consistent. However, this is something that will be interesting to explore further by looking at snails infected with multiple isolates, or snails from the field, to see if the genotypes detected change over time. Different genotypes may develop in the snail at varying rates and produce diverse numbers of cercariae, meaning the transmission potential of parasites with distinct genotypes may not be the same. Such differences have already been seen when analysing triclabendazole-susceptible and triclabendazole-resistant lab isolates of *F. hepatica* (Walker *et al.*, 2006). It is not known whether, once infected with *F. hepatica*, a snail can become infected again with another miracidia. *Lymnaea natalensis* and *Lymnaea fuscus* have been infected with multiple *F. hepatica* miracidia over time, but the infections were only four hours apart (Dreyfuss *et al.*, 2000; Dar *et al.*, 2011b). In the field, snails have been

found infected with more than one species of parasite, for example, *Calicophoron* (*Paramphistomum*) *daubneyi* and *F. hepatica* (Degueurce *et al.*, 1999; Rondelaud *et al.*, 2004; Jones *et al.*, 2015).

4.4.2 Low level of Fasciola hepatica *infection in wild caught snails from farms with known transmission*

Six of the snails (one *R. balthica*, one *P. jenkinsi* and four *S. putris*) showed evidence of *F. hepatica* based on the Novobilsky ITS-2 PCR. However, no *F. hepatica* DNA was detected in any of these samples using the Kaplan 124, McGarry RAPD or Le mtDNA PCRs. The sensitivity of the Novobilsky ITS-2 and Kaplan 124 PCRs is similar (Figure 4.5). Figure 4.8 shows that the *Plagiorchis* spp. cercariae produced a band of the expected size for *F. hepatica* using the Novobilsky ITS-2 PCR. Despite the analyses performed in section 4.3.2.3, the Novobilsky ITS-2 PCR does not appear to be specific for *F. hepatica*. Given that only the non-*F. hepatica* specific Novobilsky ITS-2 PCR yielded a product for these six snails, the most likely conclusion is that none of the snails studied here showed evidence of *F. hepatica* infection. This highlights the need to ensure that PCR primers used are specific only for the trematode of interest, particularly since the Novobilsky ITS-2 PCR has been used to report the prevalence of *F. hepatica* infection in snails (Novobilsky *et al.*, 2014).

There is evidence that *R. balthica* and *Succinea* spp. can become naturally infected with *F. hepatica* (Relf *et al.*, 2009; Caron *et al.*, 2014; Jones *et al.*, 2015). However these studies do not confirm the ability of these snails to shed and thus affect parasite transmission. A recent study has found *F. hepatica* DNA within *P. antipodarum* (synonymous to *P. jenkinsi*) snails (Jones *et al.*, 2015). Yet, again, this study did not confirm infection and transmission, and previous experiments have indicated that *P. jenkinsi* is likely to be feeding on miracidia rather than becoming infected by them (Nansen *et al.*, 1976). Nevertheless it is important to recognise the transmission potential that other snail species, in addition to *G. truncatula*, may play.

All snails collected in this study were from farms with known *F. hepatica* transmission, evidenced by infection in the definitive host. However no conclusive evidence of *F. hepatica* infection was found in any of the snail intermediate hosts studied. This fits with the low level of *F. hepatica* infection found within snails in previous studies (Crossland *et al.*, 1969; Rondelaud *et al.*, 2004; 2015); although in

some studies large percentages of snails can be found infected with *F. hepatica* (Kendall and Ollerenshaw, 1963; Hammami *et al.*, 2007; Kozak and Wedrychowicz, 2010; Novobilsky *et al.*, 2014). Whilst some of these studies are based on dissection of snails, the results of the study by Novobilsky *et al.*, (2014), which reported 81.9 % prevalence of *F. hepatica* infection in 127 snails, should be interpreted with caution given the findings in this study regarding the specificity of the Novobilsky ITS-2 PCR. The snails sampled here were collected in September, October and November. However snails tend to hibernate when temperatures fall (Boray, 1969) meaning they can be difficult to find, and the time of sampling may not have been appropriate for maximising detection of infection. The highest prevalence of *F. hepatica* infection in snails in the UK is generally reported in the summer and early autumn months (July to September; Crossland 1976; Relf *et al.*, 2011).

Since the snails collected from the field were from farms with known *F. hepatica* infection in ruminants, these results suggest a low level of infection in snails may be sufficient to sustain a high level of infection in the definitive host. This is consistent with reports in the literature of a low prevalence of infection in wild caught snails and suggests that large numbers of snails are required to assess the population structure of *F. hepatica* in field populations of snails. Alternatively there may be a reservoir of infection in another habitat or species of snail.

4.4.3 Challenges of performing these experiments

4.4.3.1 Collection, identification, maintenance and survival of snails

The snails collected from the field were found in habitats similar to those previously described (Rondelaud *et al.*, 2011). However, collection of snails is labour intensive. Although definitive species identification of the Lymnaeidae family is problematic, the morphological identification of all the snails in this study was confirmed by sequencing of the ITS-2; sequencing also allowed identification of the *Succinea* spp. to the species level.

The transmission potential, i.e. the ability of a snail to shed, is one of the most important factors (Hodasi, 1972), but maintaining snails in the laboratory to monitor shedding is also laborious. When snails are collected, if they are infected with *F. hepatica*, it is not known for how long they have been infected and so snails must be kept for 6 to 8 weeks before they can be considered not capable of shedding

cercariae. It is also difficult to know the optimal conditions required to stimulate shedding of cercariae from different snail species, other than *G. truncatula*, due to the lack of published information on this topic and the range of 'shedding' techniques described (e.g., Dreyfuss *et al.*, 1999b; El-Shazly *et al*., 2012). For studying the population genetic structure within the snail, it is preferable to use metacercariae, given the inhibitory properties found within snails which can impede PCR reactions. However, it is also important to compare infection within the snail to what is shed by the snail.

The limiting factor to determining whether a snail sheds cercariae is how long it survives. The survival rates of the snails collected from the field was 62.3 % up to 61 days. However the *G. truncatula* snails suffered a population crash whereby over five days (days 33 to 37 post collection) 27 snails died. For the experimental infections the 30 day post exposure survival rates and infection rates were similar to previous studies (Dreyfuss *et al.*, 1999b; Rondelaud *et al.*, 2014a). However, the number of snails that shed cercariae was considerably lower than other studies (Vignoles *et al.*, 2006; 2011; Sanabria *et al*., 2012; Rondelaud *et al.*, 2014b; 2014c). These parameters can also vary depending on husbandry and the number of miracidia used for infection (Dreyfuss *et al.*, 1999b; Rondelaud *et al.*, 2007b), and these factors should be considered for future studies.

4.4.3.2 Sensitivity and specificity of the PCRs used to identify Fasciola hepatica *infection within snails*

No single, simple, robust and reproducible technique is available to identify *F. hepatica* infection in the snail (Caron *et al.*, 2008). Difficulties exist with the PCR techniques because of the inhibitory factors within the snails which can reduce sensitivity (Cucher *et al.*, 2006). The use of BSA did improve the sensitivity of the Bargues ITS-2 PCR (data not shown). However, other studies only had to eliminate 7.89 % of *G. truncatula* and 8.77 % of *Radix* sp. due to inhibition (Caron *et al.*, 2014). In our study the snail region of the ITS-2 was amplified from *R. balthica* and *P. jenkinsi* in 100 % of cases, from *S. putris* in 44 % of cases and from *G. truncatula* in 55.7 % of cases. Therefore, a further limiting factor to be considered here may be the quantity of DNA produced. The amount of DNA extracted from different species of snails varied from 4.1 ngµl^{-1} to 92.25 ngµl^{-1}. Although two methods of DNA extraction were evaluated here (see 4.2.1), other methods could be considered. For

example, the use of Chelex to extract DNA from snails has been found to produce higher quantities of DNA compared to phenol extraction (Caron *et al.*, 2011).

There is also a need to balance the sensitivity and specificity of the PCRs to detect *F. hepatica*. Here the sensitivity of five PCRs to detect *F. hepatica* was evaluated (Figure 4.5). Those with the greatest sensitivity were the Novobilsky ITS-2, Robles-Perez ITS-2 and Kaplan 124 PCRs. When these PCRs were evaluated against *Plagiorchis* spp. DNA (isolated from the cercariae shed from two wild caught *G. truncatula*) the only PCR that did not generate a product was Le mtDNA. Whilst this PCR seems the most specific it was also the least sensitive of the five we evaluated. A number of other trematode species have been isolated from *G. truncatula* including *C. daubneyi, Haplometra cylindracea, Notocotylus* sp. and *Plagiorchis* sp. (Rondelaud *et al.*, 2015), and other trematodes of birds and amphibians can also parasitise snails. Some of these trematodes will have little or no published DNA sequence available, which makes it difficult to ensure PCRs are *F. hepatica* specific. Furthermore only a few of the published PCRs have been validated for use with snails.

In terms of determining transmission potential, it is possible that molecular assays can be too sensitive, for example detection of a miracidia within four hours of exposure (Kaplan *et al.*, 1995), which is well before a snail reaches the 'point of shed'. The use of multiple methods to detect infection within snails is advocated and it is suggested that sensitivity of PCRs should be determined by spiking mixtures of DNA isolated from snails with *F. hepatica* DNA to take account of the inhibitory properties (Kozak and Wedrychowicz, 2010).

Interestingly the Bargues ITS-2 PCR also amplifies a sequence from *F. hepatica* adult DNA. This is possibly to be expected given that the snail and parasite have co-evolved together. This only seems to amplify *F. hepatica* DNA when it is at a high concentration, so is not in itself useful for determination of *F. hepatica* infection. Adequate concentrations of DNA are also important for microsatellite genotyping, and it is known that a minimum of 30 metacercariae are needed to produce a MLG using the multiplex PCR (data not shown). This means that if small numbers of metacercariae are produced by a snail they may not necessarily be detected. Interestingly when the experimental snails were analysed some were positive for *F. hepatica*, but negative for the snail-specific Bargues ITS-2 PCR. However, the five experimentally infected snails, where microsatellite genotyping

was successful, were positive for both Bargues ITS-2 and for *F. hepatica* DNA (Novobilsky ITS-2 and Kaplan 124). This may give an indication of the quality and quantity of DNA extracted, and may, therefore, be a useful indicator of whether microsatellite genotyping can be performed.

4.4.4 Conclusion

Although further work is required our experimental infections have shown that *G. truncatula* can be infected with more than one genotype of miracidia. We were not able to show that snails shed cercariae of more than one genotype but there is no reason to suspect that this might not occur and therefore translates through to transmission of parasites of multiple genotypes. This observation, along with the capacity of the parasite to undergo clonal expansion within the snail, provides a means for the parasite to overcome the population bottleneck imposed by the snail, and contribute to the level of genetic diversity seen in parasites collected from the definitive host in Chapter 3. Since we found no snails definitively infected with *F. hepatica* on farms with known infection in livestock; either the level of infection in snails is low, or another reservoir of infection exists.

Although the experiments described here are labour intensive and time consuming they can produce information of value. The tools and methods developed in this chapter were primarily a ‘proof of principle’ and can be used for many experiments in the future. The availability of six clonal isolates with distinct MLGs, as well as the capacity to perform experimental infections, and observe both lab and wild caught snails for shedding of cercariae, are all valuable resources and further research is warranted.

CHAPTER 5
DISCUSSION

5.1 Unravelling the reproductive biology of Fasciola hepatica

To determine the proportion of diploid and triploid *F. hepatica* in Great Britain, the chromosomes of a statistically meaningful number of *F. hepatica* were visualised. All parasites studied were diploid. Therefore triploidy is rare, or non-existent, in field populations of *F. hepatica* in Great Britain. In addition all parasites analysed were found to contain sperm; this is in contrast to the majority of *Fasciola* sp. from Asia which are frequently described as aspermic, or triploid and aspermic (Terasaki *et al.*, 1982; 1998; 2000; Itagaki *et al.*, 1998; 2009). The presence of parasites with sperm indicates that sexual reproduction likely predominates over parthenogenesis in Great Britain. Triploids are assumed to be maintained by parthenogenesis (Terasaki *et al.*, 1998; 2000; Itagaki *et al.*, 2005a; Hanna *et al.*, 2008), and the majority of evidence suggests that triploids form following hybridisation between *F. hepatica* and *Fasciola gigantica* (Itagaki *et al.*, 1998; 2005a; 2005b; 2009; Agatsuma *et al.*, 2000; Peng *et al.*, 2009; Mohanta *et al.*, 2014). In the UK, *F. hepatica* exists in isolation from *F. gigantica*, and this infers that in countries where *F. hepatica* is the only species of *Fasciola* the parasite is also diploid and reproduces sexually. This could be further explored by determining the ploidy of *F. hepatica* in other European countries. Alongside this, determining the relative frequencies of parthenogenesis, self- and cross-fertilisation will further inform studies on the spread of anthelmintic resistance.

Following the population genetic study performed here, F_{IS} values were used to determine the proportion of self-fertilisation that occurs in the British *F. hepatica* population. This was found to be no greater than 2 %, and is in contrast to F_{IS} values reported in Spain, which indicated selfing rates > 35 % (Vilas *et al.*, 2012). The study reported here included a greater number of parasites, from a greater number of hosts and a wider range of geographical areas; and thus may be a more accurate representation of what is occurring. Alternatively there may be differences in the proportion of self- and cross-fertilisation between isolates from different countries; in addition these proportions could be influenced by selection pressures, for example anthelmintic treatment. Progeny analysis would give direct measures of self- and cross-fertilisation, as opposed to the indirect measure used here; such studies would be logistically difficult, especially for natural infections. If F_{IS} values, obtained from population genetic analyses, were combined with knowledge of the husbandry of the

definitive host, factors influencing the proportion of self- and cross-fertilisation might be identified.

5.2 Clumped transmission of parasites to the definitive host

We collected *F. hepatica* from naturally infected sheep and cattle, and genotyped them using a microsatellite panel to produce a MLG. Parasites with repeated, that is identical, MLGs were identified in 61 % of definitive hosts. Following statistical analysis it was determined that, overall, repeated MLGs tended to co-occur in the same host. It can therefore be said that there is clumped transmission of parasites to the definitive host, and substantial mixing of parasites with different MLGs does not occur prior to ingestion by the definitive host. This is supported by an additional observation made in this thesis: repeated MLGs were shared between only five pairs of animals. These pairs of animals were from the same geographical area, and in the majority of cases were known to come from the same farm, so were likely grazing similar pastures.

Clumped transmission is highly probable, since snails shed multiple cercariae at the same time (Hodasi, 1972; Dreyfuss *et al.*, 2006) and clonal expansion is known to occur within the snail intermediate host, so multiple genetically identical cercariae are produced. However, once the cercariae exit the snail, little is known about how far they travel from the snail before encysting, and where on vegetation they encyst. During the experimental infections performed here, we have observed cercariae encysting quickly, usually on the first surface they come into contact with. Although these experiments take place in a restricted area, it suggests cercariae do not travel far from the snail, and since we have detected clumped transmission in the host it is likely that metacercariae aggregate on pasture. When snails were stimulated to shed in petri dishes, there was limited evidence that (meta)cercariae aggregated together upon exiting from the snail, as groups of three or more metacercariae were found clumped together (Abrous *et al.*, 2001b). However, most of these groups were found around the walls of the petri dish, so this observation may be a product of the small area (154 cm^2) used for these experiments. Snail habitats tend to be small (Rondelaud *et al.*, 2011), this may, therefore, limit the area of pasture that becomes contaminated with metacercariae. We are developing tools to be able to detect metacercariae on pasture, which, combined with knowledge of snail habitats, can be used to characterise pasture contamination. Ultimately we hope to genotype

metacercariae from pasture using our microsatellite panel to determine if parasites of the same genotype aggregate on pasture.

5.3 Fasciola hepatica *is able to maintain a high level of genetic diversity*

Adult *F. hepatica*, isolated from naturally infected sheep and cattle in Great Britain, were found to be genetically diverse within the definitive host. This level of genetic diversity is not just a product of grazing over time, since, in this study, the genetic diversity of adult parasites collected from lambs (grazing for only one season) was not significantly different to those collected from cattle (grazing for one or more seasons). It is known that *F. hepatica* is highly fecund, and clonal expansion in the snail has the capacity to amplify the number of cercariae derived from one miracidium. However, mortality can occur at every stage of the life cycle (Ollerenshaw, 1959), thereby reducing the potential gene pool that is ultimately found in the definitive host. Indeed although we found parasites with repeated MLGs that have occurred as a product of clonal expansion, the maximum number of adult parasites with the same MLG was ten (data not shown).

Reported levels of *F. hepatica* infection in *G. truncatula* in the UK and the Republic of Ireland are low which has the potential to reduce the overall number of parasite genotypes released from infected snails (Kendall and Ollerenshaw, 1963; Crossland *et al.*, 1969; Ollerenshaw, 1971; Crossland, 1976; Relf *et al.*, 2011; Jones *et al.*, 2015). However, there may be a reservoir of *F. hepatica* infection elsewhere that contributes to genetic diversity, as there is evidence that *F. hepatica* can infect snails other than *G. truncatula* (Abrous *et al.*, 1999; Rondelaud *et al.*, 2001; Dreyfuss *et al.*, 2005; Relf *et al.*, 2009; Caron *et al.*, 2014). Snail habitats can be difficult to locate; if habitats are overlooked, the actual percentage of snails infected with *F. hepatica* could be higher. In addition snails infected with *F. hepatica* have been found in areas with no ruminant contact (Dreyfuss *et al.*, 2003), therefore there may be a role for reservoirs of *F. hepatica* in wild definitive hosts, such as rabbits (Parr and Gray, 2000).

We have shown that it was possible to infect snails, from a laboratory maintained colony of *G. truncatula*, with miracidia of distinct genotypes. Given the low proportion of snails that are infected with *F. hepatica* in Britain, if snails are infected with more than one genotype of *F. hepatica*, which all undergo clonal expansion, then this may give the parasite the opportunity to overcome the

population bottleneck imposed by the snail, and lead to the high levels of genetic diversity seen. The snails that were infected with miracidia of multiple genotypes did not shed cercariae. However, this is likely a product of a low shedding rate in the experiment overall; it is difficult to see why if a snail can become infected with multiple genotypes it would not also shed multiple genotypes. Unfortunately, this could not be assessed in the field, since none of the wild caught snails were infected with *F. hepatica*, which suggests that large numbers of snails would be required to assess the population structure of *F. hepatica*. It is known that snails can be infected with multiple miracidia, and it is suggested that the number of miracidia a snail is able to support is related to the nutrition the snail receives. It is also proposed that the calcium ion content of soil, within the snail habitat, may have an effect on the development of *F. hepatica* infection within the snail (Rondelaud *et al.*, 2004). It would be interesting to explore how the physical and chemical properties of soil might influence (i) how many miracidia can successfully infect a snail and (ii) how many cercariae are produced. Such questions could be addressed by collecting snails, and soil, from habitats with different soil types. After maintaining these snails in the laboratory, their progeny could be used for experimental infections to compare infection rates and production of cercariae.

5.4 Exploring the role of the snail intermediate host

The experimental infections of snails conducted here were really a proof of principle, and there is scope for further research. For these experimental infections, snails were exposed to different genotypes of *F. hepatica* miracidia at the same time. However, whether or not this happens in the field is difficult to know: whilst eggs will be excreted from the definitive host in the same faecal pact, they are likely to develop at different rates, and thus hatch at different times. It is not known whether once a snail has been infected with *F. hepatica* it becomes resistant to infection by other *F. hepatica* miracidia. It is known that experimentally snails can be infected with two miracidia four hours apart (Dreyfuss *et al.*, 2000; Dar *et al.*, 2011b), and in the field snails have been found infected with more than one parasite (Degueurce *et al.*, 1999; Rondelaud *et al.*, 2004; Jones *et al.*, 2015). If a snail is infected with multiple miracidia, these may develop at different rates, and thus the proportion of genotypes of cercariae that a snail produces may change overtime. This would have implications on pasture contamination, and ultimately the genotypes the definitive

host is exposed to. Using the tools developed here, these questions can begin to be answered.

Population genetic studies have also been performed on the intermediate host, *G. truncatula*, itself. Studies of *G. truncatula* from Bolivia, France and Switzerland show that these snails preferentially self-fertilise, with reported selfing rates of > 80 %. There was low genetic diversity within populations of snails, but high genetic differentiation between different populations. This is in direct contrast to the *F. hepatica* parasites studied here, and is likely a reflection of the high rates of selfing (Meunier *et al.*, 2001; 2004; Trouve *et al.*, 2003; Chapuis *et al.*, 2007). Microsatellite genotyping, based on six loci, of *G. truncatula* from the Bolivian Altiplano found that all snails had the same MLG. This lack of genetic variability could account for the high transmission rates of *F. hepatica* in humans in this area (Meunier *et al.*, 2001). Further exploration of the genetics of the snail intermediate host is warranted to determine what makes a snail susceptible to infection with *F. hepatica*.

5.5 The British Fasciola hepatica *population is panmictic*

Our analyses indicate that the British *F. hepatica* population is panmictic and showed no evidence of sub-structuring geographically or amongst parasites from sheep and cattle, and this is supported by the work of others (Hurtez-Boussès *et al.*, 2004; Vázquez-Prieto *et al.*, 2011). When collecting adult *F. hepatica* samples at slaughter it is difficult to obtain information on the history of the definitive host. The country of origin was known for sheep, and for cattle ear tag numbers made it possible to know where an animal had moved in its lifetime. However, we did not know the anthelmintic treatment history, or grazing history, of the sheep or cattle. Livestock in Great Britain are frequently moved around and between countries. Even a small amount of migration can destroy any observed population structure giving the appearance of panmixia; for example moving animals to a new farm could introduce a new population of parasites as well as exposing the definitive host to a different parasite population. This should be remembered when interpreting our results, and further analysis of parasites from, for example flocks or herds that do not buy in animals, may reveal structure not previously detected.

5.6 Implications of these findings in relation to anthelmintic resistance

We have shown that the British *F. hepatica* population is panmictic, with no evidence of structuring, and high gene flow. This means that any allele, including those that may be putatively responsible for anthelmintic resistance, can spread quickly in a population. Furthermore, since there is no evidence of structuring both geographically and between parasites from sheep and cattle, this means anthelmintic resistance could seemingly spread around the country, and between species easily; this may be compounded by animal movements. Sheep tend to be treated with anthelmintics against *F. hepatica* more frequently than cattle, and resistance to triclabendazole is more frequently reported in parasites from sheep than cattle. However, our findings indicate that resistant *F. hepatica* from sheep could be transferred to cattle. The level of diversity and genetic variation in the British *F. hepatica* population is also high, and this means there is more potential for parasites to exist that are resistant to anthelmintics.

Since multiple parasites with the same MLG have been identified this also means there is the potential for drug resistance to spread through a population if this MLG represented a parasite that was itself resistant. Furthermore, since anthelmintic resistance is likely to be a recessive trait, clumped transmission would lead to the presence of multiple parasites with the same MLG within the same host. Since the British *F. hepatica* population preferentially cross-fertilise this would increase the likelihood of these parasites reproducing with each other and passing on the trait to the next generation, compared to relying on self-fertilisation if only one resistant parasite was present. There is some evidence in our laboratory that different isolates of *F. hepatica* preferentially self- or cross-fertilise. This would be interesting to explore in relation to anthelmintic resistance, and a starting point would be to determine the proportions of self- and cross-fertilisation by progeny analysis of laboratory isolates with known resistance status.

Whilst research needs to further explore the relationship between the intermediate host and *F. hepatica,* it is also vital to consider the definitive host. Differences have been noted between the ability of triclabendazole-resistant (Oberon) and triclabendazole-susceptible (Fairhurst) isolates to establish infections in both the intermediate and definitive hosts (Walker *et al.,* 2006). These isolates were originally from different countries, and so their genetic backgrounds may

differ; yet if the fitness of parasites with different genotypes varies, this may affect the emergence and spread of genes associated with anthelmintic resistance.

5.7 Importance of our findings to future areas of research

Knowledge of the provenance, infectivity, pathogenicity, and resistance status of laboratory isolates is important (Hodgkinson *et al.*, 2013). Laboratory isolates are often not representative of what is happening in the field, and this is further exemplified by our findings. The triclabendazole-susceptible Cullompton isolate is aspermic, triploid, and assumed to be maintained by parthenogenesis; whilst some parasites of the triclabendazole-resistant Sligo isolate were found to have abnormal spermatogenesis (Fletcher *et al.*, 2004; Hanna *et al.*, 2008). However we have shown that the British *F. hepatica* population is diploid, spermic, and predominately reproduces by sexual reproduction. We have also found that the *F. hepatica* population is genetically diverse. This is in contrast to work by Walker *et al.*, (2007) on the triclabendazole-susceptible Fairhurst isolate: based on three mitochondrial loci all parasites genotyped from the Fairhurst isolate had the same haplotype. Laboratory maintained isolates of *F. hepatica* are frequently used in research, including in drug and vaccine trials (Fairweather, 2011b). However, if these isolates are not truly representative of the genetics that exist in the field then their results could be questioned.

5.8 Conclusion

We have shown that the British *F. hepatica* population is diploid, reproduction is predominately sexual, and cross-fertilisation prevails over self-fertilisation. The diploid nature of *F. hepatica* allowed us to determine the appropriate methods to calculate observed and expected allele frequencies that are fundamental to population genetic studies (Dufresne *et al.*, 2014).

A population genetic study of *F. hepatica* collected from naturally infected sheep and cattle in Great Britain was performed. This represents the largest such study in this parasite to date, and microsatellite markers are considered to be superior to a number of other genetic markers used in previous studies (Laoprom *et al.*, 2010). Across Great Britain *F. hepatica* showed no evidence of population structure, and the parasite population can, therefore, be described as panmictic with high gene flow. Some hosts contained parasites with identical MLGs suggesting clumped parasite

transmission to the definitive host, which may be due to aggregation of infective stages on pasture. The genetic diversity of *F. hepatica* is high within the definitive host and by extension on pasture. All of these factors have the potential, as discussed, to influence the spread of anthelmintic resistance in a population.

However this work also raised questions around how *F. hepatica* maintains the genetic diversity seen despite the population bottleneck caused when the parasite has to pass through the snail intermediate host. Therefore, we performed experimental infections of *G. truncatula*, and showed that snails can be infected with multiple miracidia of distinct genotypes. This may give the parasite one route to overcome the bottleneck that the intermediate host imposes. These experiments have shown the potential applications for the tools developed in this thesis. Further research is warranted to further unravel the transmission of *F. hepatica*, as well as to ultimately understand the origin and evolution of anthelmintic resistance genes in parasite populations.

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