Predicting temporal changes in *Fasciola hepatica* abundance from climatic variables

Thesis submitted in accordance with the requirements of
the University of Liverpool for the degree of Doctor of Philosophy
by
Daniel Barnaby Smith

November 2016

Abstract

Fasciola hepatica is an economically important parasite of sheep and cattle. The life cycle of this parasite includes freeliving stages and an intermediate snail host (*Galba truncatula*). In the past, several statistical models have been developed to predict how climatic factors, specifically temperature and rainfall, affect parasite abundance. However, these models were built under historic climate conditions, or for a specific geographic region, and are not well suited to predict how the epidemiology of infection might change in situations of global climatic change. The primary objective of the work described in this thesis was to develop a mathematical model of the life cycle of *Fasciola hepatica* that, for the first time, captures the seasonality of the parasite and can be used to predict parasite abundance under the varying conditions of climate change and alterations in farm management practices. To build the model, experimental studies were conducted to increase the available data on parasite development and survival in the environment.

The experimental work focused on the effects of temperature and water availability on parasite eggs and metacercarial development and survival. The survival of parasites at low temperatures suggests that overwintering of parasites on pasture is very possible. The differential equation model developed focused solely on the freeliving stages of *F. hepatica* and runs on daily temperature and rainfall (rainy day) data. The model was validated against data from published longitudinal studies and passive disease surveillance data. The limited data available to validate this type of model made it impossible to validate the model in a sufficiently robust manner. From the validation studies, it appears that the model is good at predicting the seasonality of the parasite but performs less well at predicting differences in peak abundance between years. The model predicts that, under two different greenhouse gas emissions scenarios, the abundance of *F. hepatica* will increase dramatically in future, with more than twice the number of metacercariae currently seen between August and October, and significantly more pasture contamination between June and December. The effects of dosing animals at different times of the year was also investigated, Under historic conditions, in the United Kingdom, a single dose of an appropriate helmintic treatment administered to animals in March will not reduce the number of metacercariae seen in the autumn, but a significant reduction results if an appropriate anthelmintic is administered in May. Greater metacercariae pasture contamination predicted under future climate projections will necessitate changes in farm management practices aimed at limiting transmission, which is predicted to occur earlier in the year.

Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Description
°C	Degrees Celsius
APHA	Animal and Plant Health Agency
BADC	British Atmospheric Data Centre
BTB	Bovine Tuberculosis
DEFRA	Department for the Environment, Farming and Rural Affairs
ELISA	Enzyme Linked Immunosorbent Assay
FEC	Faecal Egg Count
SICCT	Single Intradermal Comparative Cervical Tuberculin
VIDA	Veterinary Investigation Diagnosis Analysis

Acknowledgements

I would like thank my supervisors Dr Jan VanDijk, Prof Diana Williams and Dr Eric Morgan for their support and advice throughout my PhD. Dr Cyril Caminade for the temperature and rainfall data for the United Kingdom and the future climate projection data. I would also like to thank Katherine Allen for snail maintenance advice and algae (Liverpool University), Dr Hannah Rose (Bristol University) for advice on the modelling and troubleshooting problems in the coding and Jane Hodgkinson for supplying the metacercariae used in the experiments conducted at the University of Liverpool. I would like to thank Montserrat Thorp (Liverpool University) for help with data collection of overwinter survival of *F. hepatica* eggs. I would also like to thank my funding body the EU project GLOWORM which was funded under the seventh framework programme.

Chapter 1

Introduction and literature review

1.1 General introduction *F. hepatica*

Fasciolosis is a disease primarily occurring in ruminants caused by parasitic trematodes of the genus *Fasciola*, which have a complex life cycle (described below). In temperate regions, such as Europe and large parts of North America, *Fasciola hepatica*, is responsible for the majority of cases. In tropical regions, *Fasciola gigantica* is often the main cause of disease. Fasciolosis has a great impact on the farming industry due to economic loss attributed to liver damage which results in poor feed conversion, reduced milk production, weight loss, anaemia and death of animals [Brunsdon, 1967, Hawkins and Morris, 1978, Hope Cawdery et al., 1977, Kaplan, 2001, MacKintosh and Brophy, 2012], It has been estimated that, globally, fasciolosis costs the animal production industry over $2000 million a year [Spithill et al., 1999]. Within Europe, human infections are not common, but it is a zoonotic problem in some developing countries such as, Bolivia, Cuba and Egypt [Cywińska, 2016], and is recognised by the World Health Organisation as a neglected tropical disease [McManus and Dalton, 2006]. The primary source of human infection comes from freshwater vegetables, while, in Europe the most common source of infection is watercress [Ashrafi et al., 2014].

De Brie [1379] was the first to record a case of liver fluke infection in sheep, which was named liver rot at the time. The liver damage was thought to be caused by the liver itself until it was understood to be caused by a parasitic trematode acquired from infected grassland [Andrews, 1999]. There is archaeological evidence of *F. hepatica* infections in human and cattle populations as far back as 3500 BC in Germany [Dittmar and Teegen, 2003]. In addition, evidence of the snail host *Galba truncatula* inhabiting the same areas has been found from the same period.

Endemic fasciolosis can be found mostly in areas of the world that have temperatures above 10 °C for more than six months each year [Torgerson and Claxton, 1999]. In the United Kingdom, there is a peak of observed cases of disease during the autumn and a further lesser peak in spring [Torgerson and Claxton, 1999]. In warmer climates it is common to have year-round transmission of the infection, although a lack of rainfall in some months may introduce seasonality into these regions also. The infection is transmitted through the prevailing snail populations in the area. *G. truncatula* is considered the prismary intermediate host for *F. hepatica* in much of Europe including the UK and France [Dreyfuss et al., 2006]. In Europe, many other snail species have been implicated in transmission, including species from the genera *Lymnaea, Pseudosuccinea Radix and Stagnicola* [Shubkin et al., 1992, Rognlie et al., 1994]. All are found near bodies of (semi-) permanent water [Relf et al., 2009].

There are two primary clinical presentations of fasciolosis in ruminants: acute and chronic disease. Acute disease is characterised by a rapid loss of the animal's condition, lethargy, liver failure, anaemia and, in severe cases, death. This is caused by large numbers of metacercariae being ingested over a short period of time, followed by the emergent immature flukes migrating through the liver. This migration causes damage to the liver

and blood loss. Animals suffering acute infection do not excrete eggs in faeces, as the parasites have not developed to adults, this makes identification of infection by faecal egg counts (FEC) impossible. Chronic infection results in a loss of body condition along with anaemia and hardening and calcification of the liver in cattle, resulting in loss of liver function. In addition, the growth rate of sheep and cattle is reduced, as well as the production of milk in cattle and sheep may suffer poor wool growth [Torgerson and Claxton, 1999, Schweizer et al., 2005]. This is caused by mature adult fluke accumulating in the bile ducts of the liver and absorbing nutrients from the blood of the host. As mature worms are present during these infections, they can be diagnosed by the identification of *F.hepatica* eggs in the faeces of hosts [Al-Habbib, 1974].

Between 1995 and 2005, there was a marked increase in the number of reported cases of cattle diagnosed with chronic fasciolosis in the UK. Also, higher levels of cases of acute fluke infection were reported in sheep, with a very high rate of diagnosis in 2002 [van Dijk et al., 2010]. The reasons for the increase are unknown but the increase in fluke abundance may have been due to several different factors, including climate change, an increase in anthelmintic resistance and changes in pasture and grazing management. *F. hepatica* has come under increased attention as there is evidence that infection with the parasite can influence immunity to other infectious organisms and reduce the efficacy of immunological-based diagnostic tests, including the bovine tuberculosis test [Claridge et al., 2012].

Maintaining *F. hepatica* under experimental conditions for multiple generations is costly and requires infecting either a ruminant or rat/mouse host then maintaining the animal for several months until the parasite matures and begins producing eggs. The prepatent period in sheep and cattle is ˜70 days, with the development of the parasite

within the intermediate host taking around 50 days at 20 °C [Over, 1982]. The full life cycle takes 5 months or more to complete. A colony of snails must also be maintained in the laboratory to provide the infectious metacercarial stage. This requires very specific conditions and full-time attention [Vignoles et al., 2006]. If laboratory-produced isolates are maintained for too long, there is a risk of selecting parasites that are best adapted for stable laboratory conditions and are significantly different to wild isolates. *F. hepatica* eggs and metacercariae can be kept refrigerated at 4 °C for up to 12 months and still be infective, although a large proportion will become non-viable. There are relatively few isolates of *F. hepatica* available for study, and fewer than there were 20 years ago [Fairweather, 2011].

F. hepatica is not a notifiable disease in the UK and farmers are not required to inform authorities that it is present in their animals. Most cases are treated by a veterinarian or by the farmers themselves if it affects their animals regularly. This means that most of the evidence for changes in the number of fluke cases is anecdotal. In the UK, there is passive surveillance for *F. hepatica*. These data are gathered by the Animal and Plant Health Agency (APHA) and Scottish Agricultural College (SAC), either from animals that are submitted by vets or farmers for post-mortem examination, or from faecal and serum samples sent to regional laboratories for analysis. As submission of samples and carcasses to laboratories is affected by many factors, including the motivation of farmers to submit samples and familiarity with disease, fasciolosis as seen in the field may not translate immediately to the surveillance database [van Dijk et al., 2008]. Data are, therefore, patchy and may overlook areas with a high fluke burden as in these areas suspected cases are less likely to be sent in for diagnosis and the animals will just be treated. Nevertheless, data have been gathered over many years and fluctuation in the diagnostic

rate of fasciolosis may be informative. Furthermore, the Veterinary Investigation Diagnosis Analysis (VIDA) data give a good indication of the seasonality of disease throughout the UK over the years and clearly show the summer and autumn peaks of acute fluke cases in sheep. Infection is usually not evenly distributed throughout a herd, but typically some animals will be heavily infected while others will show relatively low levels of infection. If sampling protocols are sub-optimal, such overdispersion of infection can result in an incorrect estimate of the level of infection on a farm if too few animals are examined. Abattoirs also record cases of *F. hepatica* observed in the livers of slaughtered animals, although in the UK there is currently no central database of these records.

1.2 The importance of *F. hepatica*

With the costs to the farming industry estimated at two billion dollars per annum worldwide, *F. hepatica* is not only an animal health issue but also a serious burden on the agricultural economy [Spithill et al., 1999]. The greatest economic loss is caused by the reduction in animal productivity, but there are additional costs incurred by the farming industry including paying for treatment, diagnosis and veterinary care. It was estimated that *F. hepatica* infection in dairy cattle resulted in a median loss of 299 Euros per animal [Schweizer et al., 2005]. Historically, acute liver fluke infection resulting in death was confined to sheep, which may be more susceptible to liver damage. In recent years, several cases of cattle deaths caused by acute fluke have been reported [Adrien et al., 2013]. This may reflect an increased abundance of infectious metacercariae on pasture.

F. hepatica can suppress the host immune system which affects the performance of some diagnostic tests. Bovine tuberculosis (bovine TB), caused by *Mycobacterium bovis*, is a serious infectious disease of cattle, which can also affect humans. According to EU regulations, all cattle infected with bovine TB have to be removed from a herd and culled. The primary diagnostic test used as part of the UK's eradication programme for bovine TB is the single intradermal comparative cervical tuberculin (SICCT) test. Flynn et al. [2007] raised the question of fasciolosis causing false negative bovine TB skin tests. Claridge et al. [2012] showed that there is a strong association between a negative bovine TB skin test and infection with *F. hepatica*, probably due to the effect of liver fluke on the immune system. This reduction in the sensitivity of the primary diagnostic tool for *M. bovis* could seriously undermine the UK's control programmes for bovine tuberculosis.

1.3 Life cycle of *F. hepatica*

The first published observation on liver fluke was made by De Brie [1379], but it was not until 1914 that the life cycle was fully understood [Andrews, 1999]. The life cycle of *F. hepatica* is complex, as the environmental stages are microscopically small and are difficult to detect outside of a laboratory.

1.3.1 Parasite stages found in the definitive host

Metacercariae are ingested by the definitive host and pass down the digestive tract until they reach the small intestine where they lose their protective outer shell (excyst). The first phase of excystment is an active process triggered initially by high carbon dioxide levels and temperature close to 39 °C then the second phase is triggered by bile to complete the process [Dixon, 1966]. The immature parasites then migrate through the intestinal wall into the peritonial cavity and find their way to the liver. Upon reaching the liver, the immature fluke move through the liver parenchyma towards the bile ducts. During this time the immature parasites grow significantly. Migration through the liver causes extensive haemorrhage and fibrosis. Once inside the bile ducts the parasite develops fully and begins to produce unembryonated eggs. *F. hepatica* is hermaphroditic which means it can self-fertilize as well as cross-fertilize. Adult flukes have been reported to live up to 11 years in sheep and produce up to 20,000 eggs per day [Happich and Boray, 1969b].

1.3.2 Development of eggs to miracidia on pasture

Unembryonated eggs are deposited into the environment through the host's faeces. The eggs may undergo partial development while inside moist faeces, but full development will not occur until the eggs are free from the faeces [Rowcliffe and Ollerenshaw, 1960].

This lack of complete development within the faeces may be be due to limited oxygen availability. The eggs are washed from the faeces as they disperse and are broken up by rain and insect activity. After liberation, the eggs complete their development on pasture and in water bodies. The fully developed miracidium will remain inside the egg until stimulated to hatch. Light has been implicated in stimulating egg hatching [Rowcliffe and Ollerenshaw, 1960]. The hatched miracidia are non-feeding stages and, therefore, they have limited energy reserves. The hatched miracidia can remain active for up to 24 hours under cool conditions (10 °C) [Shaw and Simms, 1930]. The miracidia then penetrate the snail through a mechanical boring action [Wilson et al., 1971].

1.3.3 Parasite stages in the intermediate host

After penetration, the miracidium develops into a sporocyst which migrates through the snail's digestive gland. In the digestive gland, the sporocyst develops into a ball of germinal cells that become the daughter rediae. The rediae also develop into germinal balls which become the final larval stage of the parasite, the cercariae. Krull [1941] demonstrated that snails infected with a single miracidium could produce multiple metacercariae. This clonal multiplication inside the snail, with one miracidum capable of turning into 300 metacercariae [Vignoles et al., 2006], increases the levels of pasture contamination, leading to a higher risk of infection for the mammalian host. The cercariae remain inside the snail until stimulated to shed, at which time a mass release will occur. In the laboratory, mass release of cercariae is induced by exposing the snails to a temperature shock by exposing them to a temperature of 10 °C and then allowing the temperature to rise again to room temperature. Snails shed metacercariae in waves with several days interval between releases. If the snail survives this initial shedding of cercariae, it is likely to continue

shedding for several days or even several months [Dreyfuss and Rondelaud, 1994].

1.3.4 The infective metacercarial stage on pasture

Cercariae are motile and can swim freely in water although their movement outside water is limited [Vignoles et al., 2006]. Within two hours of emergence from the snail, the cercariae will rest on a surface, such as a blade of grass, where encystment will occur. The cercariae will lose their motile tails and become metacercariae. The protective shell consists of two layers, a tanned protein and and carbohydrate [Dixon, 1967] which provide protection for the parasite against desiccation. As soon as encystment occurs, the metacercariae become infective. The metacercariae can survive for several months on pasture under high relatively humidity conditions until they are ingested by the definitive host [Olsen, 1947]. The infectivity of the metacercariae diminishes over time, meaning that ingested fesh metacercariae are more likely to establish infection in the mammalian host than aged metacercariae.

1.4 Biology of *F. hepatica*

1.4.1 *F. hepatica* egg

The appearance of eye spots in the miracidia within the egg marks the completion of development and these are visible under light microscopy [Walker et al., 2006]. It is normal to observe some variation in the time between embryonation and eggs hatching, even among eggs kept under the same environmental conditions. This delay between embryonation and hatching can be up to two months [Shaw and Simms, 1930, Thomas, 1883]. This could be an evolutionary adaptation to ensure that all the miracidia are not killed if there is a rapid adverse change in environmental conditions.

There are varying reports on the number of eggs produced by adult flukes, this can

range from 4,000 to 50,000 eggs per day [Happich and Boray, 1969b, Valero et al., 2002, Walker et al., 2006]. The egg production per fluke is likely to be density dependent. In sheep infected with fewer than 50 flukes, each fluke will produce an average of 25,000 eggs per day [Happich and Boray, 1969b]. The greater the fluke burden within an individual animal, the lower the number of eggs typically produced by each individual fluke [Happich and Boray, 1969b]. This phenomenon could be a result of increased competition for nutritional resources. Happich and Boray [1969b] found that the total daily egg output from sheep with light infections was around 0.5 million eggs. Sheep with moderate or heavy chronic infections shed between 2.0 and 3.5 million eggs daily. Walker et al. [2006] studied the effect of differences in the fecundity between isolates and found that the Oberon isolate was twice as fit as the Fairhurst isolate.

The time taken for *F. hepatica* eggs to complete their development is strongly influenced by environmental temperature. This relationship was first recorded by Thomas [1883] but experiments conducted by Al-Habbib [1974], Rowcliffe and Ollerenshaw [1960] quantified the relationship between temperature and development for the first time. Thomas [1883] established that eggs maintained at 37 °C do not undergo development and that temperatures between 23 °C and 26 °C resulted in the shortest development times. Those kept at these temperature developed in two to three weeks, whereas eggs kept at 16 °C took two to three months to develop. These results were supported by work in later publications [Rowcliffe and Ollerenshaw, 1960, Al-Habbib, 1974].

Luzón-Peña et al. [1992] stated that the overwintering of eggs deposited in autumn makes only a minor contribution to the summer infections of the following year when compared to the eggs that are excreted from mid-winter onward. Under summer conditions, the longer the time spent in faeces, the higher the egg death rate [Luzón-Peña

et al., 1992]. The survival of *F hepatica* eggs is influenced by several factors, including moisture and temperature [Al-Habbib and Grainger, 1983]. Eggs that have not undergone embryogenesis can survive freezing temperatures, but eggs containing partially developed embryos do not develop when the temperature is raised [Shaw and Simms, 1930]. Temperatures substantially below zero degrees Celsius have a detrimental effect on the survival of *F. hepatica* eggs. Eggs kept at -15 °C for 24 hours do not develop [Boray, 1969]. Eggs will tolerate cold conditions and fluctuating daily temperatures between -6 °C and 10 °C [Luzón-Peña et al., 1991]. *F. hepatica* eggs can develop at pH levels between 4.2 and 9, but levels above pH 8 cause the development to be prolonged [Rowcliffe and Ollerenshaw, 1960]. Gaasenbeek et al. [1992] found that 80% of *F. hepatica* eggs in slurry for one month would non viable and therefore slurry spreading did not contribute to the levels of infection. Eggs are susceptible to desiccation during their whole development [Roberts, 1950, Rowcliffe and Ollerenshaw, 1960]. The primary stimuli that results in the hatching of *F. hepatica* eggs is bright light. The hatching of embryonated eggs can take place after approximately one hour the appropriate stimulus [Shaw and Simms, 1930]. Under laboratory conditions, incandescent light more easily promotes hatching than fluorescent light suggesting that specific wavelengths of light are important in stimulating hatching [Hussein et al., 2010].

1.4.2 *F. hepatica* miracidium

After hatching, the motile miracidial stage of *F. hepatica* is approximately 140μm by 75μm in size [Hussein et al., 2010]. Miracidia actively seek out a host of the lymnaeid family of snails [Nansen et al., 1976]. However, in the absence of a preferred host, the miracidia will attempt to infect snails of other families such as radix [Relf et al., 2009], although

development rarely occurs in such snails [Kendall, 1965]. Nansen et al. [1976] showed that the miracidia have the ability to chemo-locate snails in their vicinity. Miracidia are attracted to a suitable snail host from a distance of up to 15cm. *G. truncatula* has been shown to exert a stronger attraction than other snails [Christensen et al., 1976]. Some surface moisture is crucial for the miracidia to move to a snail host [Gettinby and Byrom, 1991]. Infection of the snail host is more likely to occur when water is present, as snails enter a period of aestivation under drought conditions. A single snail can become infected by multiple *F. hepatica* miracidia, as a current infection does not prevent subsequent miricidia from attaching and penetrating the snail's foot [Christensen et al., 1976]. If the snail survives the damage done by these multiple miracidial infections, these can lead to a greater number of cercariae ultimately being produced by the snail [Nice, 1979].

The life-span of miracidia is relatively short compared to that of the egg and the metacercarial stages. Miracidia are non-feeding and rely on a non-replaceable energy reserve for survival. At room temperature (20 °C), the majority of miracidia survive for about eight hours, few survive beyond 24 hours [Shaw and Simms, 1930, Al-Habbib and Grainger, 1983]. If the miracidium fails to penetrate a snail within the limited life-span of about 5 hours at 27 °C or up to 20 hours at 10-13 °C (Table 1.1), becomes moribund [Pantelouris, 1963]. Under warm conditions, the miracidia are more active and exhaust their energy reserves more rapidly [Al-Habbib and Grainger, 1983].

There are many factors that affect the success of miracidial infection of the intermediate snail host, including moisture and chemical stress on the snail prior to exposure to miracidia, both of which increase the probability of infection [Abrous et al., 2001]. Prah and James [1977] showed that for *Schistosoma mansoni*, the age of the miracidia had a large effect on the percentage of snail infections, older schistosome miracidia had

Table 1.1: Survival of miracidia at constant temperatures taken from Al-Habbib and Grainger [1983]

Temperature (°C)	Mean Survival (hours)	Standard Error	Number of miracidia studied
10	22.71	0.84	40
15	16.42	0.51	40
20	10.29	0.32	40
26	7.60	0.27	40
30	5.78	0.22	40

a lower probability of infecting a snail. Temperature does not markedly affect the rate of *F. hepatica* miracidial infection of the snail. Only a 22% increase in infection rate was observed when temperatures were increased from 10 to 20 °C [Wilson and Taylor, 1978]. In comparison to the other external life stages of *F. hepatica*, the miracidia spend a very short time exposed to environmental conditions.

1.4.3 *F. hepatica* inside the intermediate host

Snails exposed to multiple miracidial infections produce more cercariae than those exposed to a single miracidium [Kendall, 1949, Lee et al., 1995]. Shedding of cercariae begins approximately 6 weeks after miracidial infection [Krull, 1933] and can continue for more than six months [Shaw and Simms, 1930]. Once a snail is infected, it does not clear the infection during its life time [Kendall, 1965]. Active snails rarely survive more than one year [Gettinby and Byrom, 1991] although, they can aestivate for periods of up to a year if environmental conditions are unfavourable [Kendall, 1949]. In this way, snails are resistant to drought conditions, surviving more than 4 months without water, thwy can also survive freezing temperatures as low as -8 °C for several months [Schweizer et al., 2007]. *G. truncatula*, normally a mud-dwelling snail not favouring water coverage, can also survive 6 weeks or more submerged in water [Kendall, 1949]. Aestivation is more

commonly stimulated by dry conditions as opposed to high temperatures [Luzón-Peña et al., 1995].

The time from *F. hepatica* infection of the snail to first cercarial release from the snail is strongly influenced by temperature. It takes approximately 80 days at 15 °C and 20 days at 30 °C for the parasite to fully develop within the snail and be ready to shed [Gettinby and Byrom, 1991]. The optimum temperature for shedding is between 20 and 27 °C with fewer cercariae shed at temperatures below 20 °C [Lee et al., 1995] and no cercarial shedding occurs at temperatures below 10 °C [Kendall and McCullough, 1951, Luzón-Peña et al., 1995]. The number of cercariae released from infected snails is highly variable, with some studies finding that snails shed up to 44 cercariae per day [Krull, 1933] although there can be significant variation between snails and over time [Vignoles et al., 2006]. The proportion of snails infected with *F. hepatica* within a population has generally been found to be less than 20% of the snails examined [Rondelaud and Dreyfuss, 1997, Schweizer et al., 2007, Relf et al., 2011].

There are differing reports on the survival of infected snails compared to uninfected. Rondelaud et al. [2004] reported that *G. truncatula* snails infected with *F. hepatica* tend to survive longer than uninfected snails. Another study found that snails infected by miracidia of *F. hepatica* had a lower survival rate compared to uninfected snails [Gutiérrez et al., 2000] when kept in isolation. Infection by the parasite can have other adverse effects on the snail, including tissue damage, gigantism [Hodasi, 1972] and greatly lowered fecundity or total castration [Hodasi, 1972, Dreyfuss et al., 1999, Graczyk and Fried, 1999, Gutiérrez et al., 2000].

Individual snails kept in separate trays appear to feed more extensively, this can result in better nutritional status and more rapid growth of the snail which results in more

internal damage by the parasite [Hodasi, 1972]. Snail nutrition plays an important role in determining the development rate and the number of cercariae shed. Snails with better nutrition produce cercariae in a shorter time period than those with poor nutrition [Kendall, 1949, Lee et al., 1994, Rondelaud et al., 2004]. In addition, large snails can support a greater production of cercariae than smaller snails [Boray, 1969, Kendall and Ollerenshaw, 1963].

1.4.4 *F. hepatica* cercariae and metacercariae

There is variation in some characteristics of metacercariae produced from different mammalian hosts. Although the metacercariae that result from eggs shed from different mammalian hosts do not show different infectivity or pre-patent periods [Dixon, 1964, Valero and Mas-Coma, 2000], experimentally, lambs infected with a bovine strain of *F. hepatica* showed twice the number of adult flukes establishing in the liver compared to infections of ovine origin for a given infecting dose [Knight, 1978]. The number of metacercariae ingested by cattle and sheep can be diluted by rapid pasture growth [Ollerenshaw, 1971]. The intermediate hosts of *F. hepatica* are semi-aquatic and are found near water, in times of summer grass, shortage the sheep will graze nearer water, increasing their risk of acquiring infection [Khallayoune et al., 1991].

Cercariae attach to vegetation, where they shed their tails and encyst, becoming the infective metacercarial stage. Metacercariae become infective to cattle and sheep within 24 hours of encystment [Mas-Coma and Bargues, 1997]. The infectivity of metacercariae slowly decreases over time, with older metacercariae less likely to establish an infection in cattle and sheep [Valero and Mas-Coma, 2000]. The survival of metacercariae is partly determined by the availability of water, metacercariae which are fully submerged in wa-

ter tend to survive longer than those under high humidity conditions [Luzón-Peña et al., 1995]. Indeed, in a study in the Netherlands, it was found that there was a positive correlation between metacercarial availability and rainfall [Gaasenbeek et al., 1992]. Warm summer temperatures of 25 °C and above have a negative effect on the survival of metacercariae [Luzón-Peña et al., 1995]. In work conducted by Boray [1969], it was found that metacercariae kept on moist filter paper were infective after 92 days at -2 °C.

1.5 Epidemiology of *F. hepatica*

As described above, the abundance of the parasite and, therefore, the prevalence of disease within a region, is determined by a combination of factors including environmental, temperature and rainfall and geographic conditions, such as soil type and pH [McCann et al., 2010] and farm management practices [Torgerson and Claxton, 1999]. An appropriate snail host must be available on pasture on which animals have been shedding eggs. In temperate regions, it is only possible for one generation of *F. hepatica* to occur per year. Due to weather and temperature conditions in the UK, acute disease can be seen between October and December, subacute disease between October and January and chronic disease between January and April [Abbott et al., 2012] (Table 1.2). The parasite can also survive over winter inside the snail and this can lead to a second spring peak of acute cases of disease, though this rarely occurs in the UK [Luzón-Peña et al., 1994, McMahon et al., 2016].

The population of snails in an area can be significantly reduced either by human intervention, such as draining fields, or by unfavourable climatic conditions, which can cause the soil to dry or flood. Once conditions improve, the snails can recolonise the area extremely rapidly. The snails require an habitat with an acidic pH, soft water and a clay

Table 1.2: The pattern of *F. hepatica* development in different geographic regions from Abbott et al. [2012]

Climatic zone	Parasite stages on pasture	Generations per year	Infection of host	Disease outbreaks
Cool temperate, cool continental	Summer	0.5 - 1	Late Summer - Autumn	Autumn and Winter
Warm temperate, warm continental	Late Spring - early Autumn	1 - 2	Late Spring - mid-Winter	Late Summer - Spring
Sub-tropics	Early spring - late autumn	2 - 3	Early Spring - mid-Winter	All year round
Tropics	All year round	3 - 4	All year round	All year round

based substrate. They tend to inhabit area which peripheral to standing water bodies such as ponds, steams and ditches [Rondelaud et al., 2011]. Ducheyne et al. [2015] predicted the West of Ireland and the West of the UK had the highest probability of suitable conditions for *F. hepatica*. Later modelling by Caminade et al. [2015] indicates that the high risk areas will expand Eastward not only in the UK but across Europe. Several wildlife species, including rabbits and deer, have been implicated in maintaining *F. hepatica* infection in areas without farmed ruminants [de Souza et al., 2002, Schweizer et al., 2005, Vignoles et al., 2006]. Without treatment, adult fluke will live in the mammalian host for many years and will continue to shed eggs throughout their lifetime. This means that the fluke can tolerate several unfavourable years but resume development as soon as conditions allow it.

1.6 Diagnostic methods

Historically, the primary method of identifying *F. hepatica* infection was the microscopic detection of eggs in faeces. The test is conducted after sedimentation or salt (zinc sulphate)

flotation of eggs to separate them from faecal matter. This test can be conducted on individual samples or on a composite sample from several animals in order to detect the presence of infection within a herd of flock. The method is effective at identifying current, patent, infections in animals, although immature infections before the parasite has matured go undetected by the faecal egg counting method. A trained individual can accurately identify and differentiate *F. hepatica* from other helminth eggs. The specificity of this test is high, but varies with the experience of the person performing the test. The sensitivity is determined by the quantity of faeces examined, with >30g of cattle faeces yielding a sensitivity of up to 90% [Rapsch et al., 2006]. There is currently no gold standard test for *F. hepatica* [Mazeri et al., 2016], however, detection at slaughter by liver necropsy have very high sensitivity (99%) and specificity (98%).

More recent advances in diagnostic methods have resulted in the development of other detection methods, such as the copro antigen ELISA (cELISA) which tests faecal samples for the presence of excretory-secretory antigens and serum ELISA (sELISA) tests which tests serum samples for antigens. Mazeri et al. [2016] found that the cELISA had a sensitivity of 0.77 and specificity of 0.99 where as the sELISA had a less reliable estimate of sensitivity varying between 0.7 and 0.94 and a specificity of between 0.76 and 0.89. ELISA based diagnostics are used on both mammalian faeces and bulk milk tanks in dairy cattle to assess the level of infection within a herd [Charlier et al., 2014b].

PCR and Loop-mediated isothermal amplification (LAMP) have been used to detect fasciolosis in the faeces of experimentally and naturally infected sheep [Martínez-Valladares and Rojo-Vázquez, 2016]. This method has advantages over PCR diagnostics as a standard PCR takes 3 hours compared to just over 1 hour for the LAMP assay. Both the the standard PCR and the LAMP assay were able to diagnose experimentally infected

animals from the first week post infection [Martínez-Valladares and Rojo-Vázquez, 2016].

To detect infection within snails, these are brought into the laboratory and observed to shed cercariae. The detection of immature infection within snails by dissection is challenging as it requires the observation of serial sections of fixed and stained tissues. A real time PCR test has been developed for detecting infection inside the snail before cercarial release [Rognlie et al., 1994, Shubkin et al., 1992, Relf et al., 2009]. Infection can be detected with the snails host they are rarely examined to identify infection when considering specific on farm control programmes as the snails are challenging to locate and a low proportion carry infection [Anderson, 1978]. However larger scale control programmes have examined snails for infection longitudinally for estimates of prevalence [Mage et al., 2002].

1.7 Control of *F. hepatica*

There are a number of approaches available to control *F. hepatica* infection in farmed ruminants. These include treatment of affected animals, modifying the environment to drain standing water or to keep animals away from boggy areas [Roberts and Suhardono, 1996] and, in recent years, development of vaccines [McManus and Dalton, 2006] although a vaccine is not currently commercially available. Statistical models have been used to identify high risk months when high levels of infection are expected on pasture in order to allow farmers to take appropriate countermeasures [Ollerenshaw and Rowlands, 1959].

Adult fluke can shed between 20,000 and 50,000 eggs each per day, and animals can be infected with large numbers of fluke which can result in the release of millions of eggs onto pasture [Happich and Boray, 1969a, Walker et al., 2006]. Due to the clonal multiplication of the parasite inside the snails, as well as the limited habitat for the snail,

snails are a potential target for control programmes. Historically, molluscicide application, along with draining the land, resulted in a reduction of fasciolosis cases [Ollerenshaw, 1971]. This process had be repeated every year, as recolonisation by the snails was rapid. The application of molluscicide early in the transmission season reduced the amount of infection experienced by animals throughout the year [Ollerenshaw and Rowlands, 1959]. There are currently no molluscicides licensed for use in the UK currently and farmers, under EU environmental stewardship schemes, are encouraged to maintain boggy areas on their land and graze them to help wildlife conservation efforts. It is now more common to see farmers restricting herd access to these high-risk areas for infection with the use of fences.

Treatment

Table 1.3: The efficacy of flukicides for treatment of cattle [MacKintosh and Brophy, 2012]

Flukicide	Age of fluke (weeks)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Albendazole Oxyclozanide										50-70%		80-99%		
Nitroxynil Closantel							50-90%			91-99%				
Triclabendazole (TCZ)		99-99%		99-99.99%										

Triclabendazole is widely used for the treatment of fasciolosis in ruminants as it targets very immature fluke right up to adults. In dairy cattle, the choice of flukicide is influenced by the period of milk withhold, triclabendazole has a 50 day milk withhold, meaning that it is usually only used at drying off. Albendazole and oxyclozanide, although they are only effective against older fluke, have milk withholds of 60 hours and 72 hours respectively.

Animals are often treated at specific times of year or point in the production cycle without testing the animals for infection.

Current treatments are focused on treating the clinical effects of the disease as opposed to interrupting transmission. The majority of the infected animals would have been shedding eggs in their faeces onto the fields throughout the summer months, leading to the propagation of disease in the following spring. Triclabendazole is currently the only drug effective against immature fluke, other available drugs on the market, such as Nitroxynil and Closantel, are only effective in killing fluke that are six weeks or older (Table 1.3). This means that, even after treatment, if metacercariae are present at pasture and reinfection occurs, some animals will still be capable of shedding eggs that year.

Drug failure and resistance

The faecal egg count reduction test (FEC RT) is conducted by comparing the faecal egg counts before and after treatment [Coles et al., 1992, Morgan et al., 2013, Hanna et al., 2015] and can be used to detect resistance developing to antihelmintics. However, this has the limitation of only showing the effect on adult fluke. There are signs of resistance developing towards triclabendazole, which manifests initially as a failure to kill the youngest immature fluke [MacKintosh and Brophy, 2012]. This is confirmed by eggs being detected in faeces earlier than would be expected if the infection had been fully cleared [Abbott et al., 2012]. Triclabendazole resistance has been reported in several countries, including, Spain and the Netherlands [Moll et al., 2000, Álvarez Sánchez et al., 2006], as well as Australia, Ireland, Scotland, Wales [Álvarez Sánchez et al., 2006]. Apparent treatment failure can be due to liver damage preventing the fluke metabolising the flukicide, or inaccurate or incorrect dosing which may be misinterpreted as drug resistance [Abbott et al., 2012,

MacKintosh and Brophy, 2012]. At present resistance has not been observed to nitroxynil or closantel [Hanna et al., 2015].

1.8 Predictive models of *F. hepatica* abundance

The stages in the liver fluke life cycle where the parasites are exposed to environmental conditions are very important for modelling transmission as they allow temperature and rainfall data to be used to predict areas that will support transmission. The first model to predict the transmission of *F. hepatica* was created by Ollerenshaw and Rowlands [1959] and used temperature and rainfall data to predict fluke burden in sheep on the island of Anglesey in North Wales. This model has been expanded to predict the expected burden over the whole UK and is still the primary model used. The equation which governs the Ollerenshaw model (Equation 3.3), generates an Mt value, which equates to the predicted contributions of month to the expected fluke burden in autumn. Mt values <300 predict little or no disease observed, values >300 and <400 predict occasional animal losses, Mt values >400 predict that disease will be highly prevalent with many animal losses, whereas >475 predicts a serious epidemic of fasciolosis.

$$Mt = N(R - PE + 5) \tag{1.1}$$

Mt = amount of disease expected, N = number of rain days, R = precipitation and PE = evapotranspiration rate calculated by the Penman method [Penman, 1948]. Fox et al. (2011) applied the Ollerenshaw model to predict the effect of future climatic conditions in the UK (UKCP09). It was predicted that, over the next 60 years, the risk of fasciolosis in the UK will be higher than it has been in the past, for all regions, based on expected patterns of climate change. Currently *F. hepatica* development is limited by low tempera-

tures and dry conditions, with the predictions that future climate conditions will produce warmer and wetter areas, which will increase the ability of the parasite to survive and develop in the environment. However, the Fox et al (2011) paper assumes that *Fasciola* epidemiology and the seasonal contribution of the months of the year to fluke abundance will remain constant whereas this is unlikely to be the case [van Dijk et al., 2008, 2010].

Other models created to predict the risk of fasciolosis have also focused on the effect of water availability during certain months of the year as the primary determinant of infection risk [McIlroy et al., 1990, Malone et al., 1998]. Both of the models created by Ollerenshaw and Rowlands [1959] and Ross [1970] used pre-determined months of the year to calculate the predicted risk of fasciolosis. These months were chosen, based on the known epidemiology of the parasite at the time the models were created. On Anglesey, the Ollerenshaw model used temperature and rainfall data between May and October, as the average temperature was above 10 °C [Ollerenshaw and Rowlands, 1959].In contrast Stormont "Wet day" forecasting system for fasciolosis uses June to September [Ross, 1970]. In Ireland, the average temperature in May was below 10 °C and was, therefore, removed and October was removed due to epidemiological information suggesting that it did not contribute to disease [Ross, 1970]. McIlroy et al. [1990] in Ireland used the months of June to May to predict annual outbreaks. All of these models have the same drawback when trying to predict the risk of fasciolosis into the future, they all rely on a set number of months contributing to the final disease outcome. If the average temperature rises and there is a longer transmission season, then these models will not be able to capture the change in conditions. Also, they only predict total burdens around autumn, without giving any explicit information on when pasture may become dangerous for animals to graze. Therefore, none of these models can facilitate the targeted application of flukicides

during risk periods in space and time, which appears urgently needed to preserve drug efficacy. It appears that temporally explicit models, able to accurately predict risk periods, are urgently needed. These could improve current fluke control progammes by, for the first time, quantifying the effect of drug applications at certain times of the year on subsequent metacercarial abundance.

Both the Ollerenshaw and McIlroy models predict the risk of fasciolosis over either a monthly time scale or for an entire season. Both can be very powerful tools but they have several limitations. These include that the models rely on climatic conditions in specific months to predict the prevalence of fasciolosis later in the year. If climate change in the UK results in a general rise in temperature, then the range of months in which the parasite can develop is likely to expand and the appropriateness of these models will become questionable and they will probable need to be modified. These models focus solely on autumn fasciolosis so if the parasite becomes more abundant at different times of the year, then the additional 'risk' will not be captured. In addition, neither of these models is designed to take account of changes in farm animal practices, such as different strategies for treating animals with antihelmintics at different times of the year also different drugs and alterations in grazing practices such as housing of animals or restricting access to high risk areas.

1.9 Aims and Objectives

The overall objective of the work undertaken in this thesis is to predict the temporal variation in risk for farmed ruminants of acquiring infection with *F. hepatica*, based on climatic variables. Climatic variables are often used when constructing models to predict variations in the density of parasites with environmental life stages. Such modelling can

be undertaken using various approaches. These include models exploiting geographic information systems (GIS), statistical models and mathematical models. GIS models for *F. hepatica*, for example, relate the prevalence of infection in livestock in specific locations to environmental conditions and extrapolate the prevalence to areas where there are data on environmental conditions but no infection data. Statistical models seek to relate outcome variables, in this case fluke prevalence, to explanatory variables such as climate (e.g. temperature and rainfall), soil type and farm management practices [McCann et al., 2010]. This method is often used to identify the most important factors correlated with prevalence in a given area. Mathematical models for *F. hepatica* have been created using a variety of different approaches. All of the published models incorperate climatic data to predict variation in the prevalence of disease on farms year to year. These models often build on statistical modelling to determine which variables are most important to include in the modelling process.

The primary aim of this study was to create a differential equation model that predicts the abundance of *F. hepatica* on pasture throughout the year using environmental variables. Currently, the available quantitative data on the survival and development of the environmental stages of *F. hepatica* are lacking in some key areas. In addition to creating the model, data will be gathered on the effect of temperature and water availability on the development and survival of the environmental stages of *F. hepatica*. Experiments on *F. hepatica* eggs and metacercariae will be conducted under laboratory conditions and eggs will also be used in field experiments, and the effects of temperature and moisture mathematically described. Crucially, the model, for the first time, attempt to include the effects of temperature and rainfall on the snail intermediate host. This was important because climate change will have an effect on the snail population as well as the free-living

stages of the parasite and the resultant effect on both is not predictable using established models. The model was validated using published data as well as VIDA disease data which is a passive surveillance database of animal sent to the APHA for diagnostics. The validated model was subsequently be used to predict the effects of climate change on the seasonal abundance of the infective stages of the parasite as well as the effect of the seasonal application of anthelmintic flukicides, now and in the future.

Chapter 2

The effect of temperature and water availability on the development and mortality of the free-living stages of *Fasciola hepatica*

2.1 Introduction

Studies on the life cycle of *F. hepatica* have established the relationship between environmental conditions and the abundance and proliferation of *F. hepatica* in the environment. Such studies have shown that areas with regular rainfall and annual daytime temperatures between 10 °C and 25 °C are ideal for parasite survival and development [Rowcliffe and Ollerenshaw, 1960]. Most studies on the survival and development of the individual external life stages of *F. hepatica* were published before 1970 and there are parts of the life cycle that have been less well studied with respect to the development and survival under different environmental conditions. For example, all the data on the miracidial stages have been generated in laboratory experiments rather than from *in-situ* observations in the field.

Most experimental studies on the effect of temperature on the development and survival of *F. hepatica* have been conducted between 9 °C and 35 °C [Boray, 1969, Ollerenshaw, 1971, Al-Habbib, 1974, Al-Habbib and Grainger, 1983]. The effect of low temperatures on the survival and development of eggs has not been as widely studied, and the small number of studies published have inconsistent results. The development of both the parasite and the snail host was most rapid at 26 °C, with the lower temperature limit for snail activity and parasite development of 10 °C [Roberts, 1950, Rowcliffe and Ollerenshaw, 1960, Ollerenshaw, 1971, Al-Habbib, 1974].

The effect of varying temperatures on the development of *F. hepatica* eggs from Peru was studied by Claxton et al. [1999], who found that there was no statistical difference in development time between eggs held at a constant average temp of 11 °C and those kept in conditions where the temperature was changed twice a day from -1.7 °C and 23.9 °C. Some of the eggs exposed to varying temperatures failed to hatch and there were some problems reported in controlling the temperature.

F. hepatica cercariae emerge from the snail host and subsequently encyst on herbage as metacercariae. Boray and Enigk [1964] conducted a series of experiments on the effect of temperature on the survival of metacercariae kept in water between -10 °C and +35 °C. The survival and infectivity were assessed using two methods, first, by a visual examination of the metacercariae followed by attempting to infect five mice with five metacercariae each. The mice were then dissected at post mortem to look for signs of establishment of infection within the liver. They found that metacercariae were infective for at least 28 days at temperatures between -10 °C and +30 °C. The longest survival time was seen at 10 °C.

Once established on vegetation, metacercariae may be susceptible to desiccation. The

degree to which this happens is determined not just by the humidity of the air but also by the micro-climate created by the grass blades; these may mask the effects of low air humidity. The relative humidity of the environment appears to play an important role in determining the length of time the metacercariae will remain viable. Boray and Enigk [1964] demonstrated that an increase in the relative humidity from 80% to 90% resulted in an almost 4-fold increase in average survival time, from 31 to 122 days. However, relative humidity data for grass level are not available for climate modelling purposes and it is also unclear how air humidity translates to the humidity experienced by metacercariae. Therefore, with rainfall data readily available, it is important to measure the effect of rain events, and periods of drought, rather than the effects of relative humidity, on free-living stages.

Research questions

The purpose of the experimental work conducted in this chapter was to increase the available data on the effect of temperature and water availability on the survival and development of *F. hepatica* free-living stages, in order to construct a mathematical model for parasite development. Several areas were identified as lacking quantitative data, including the effect of cold and freezing temperatures on egg survival. This is particularly important for temperate areas of the world where eggs may be able to survive over winter on pasture. In this chapter, experiments to investigate the effect of temperatures between -8 °C and 26 °C on egg development under laboratory conditions are described, as well as the potential for eggs to survive over winter in the environment. Temperatures in previously researched temperature ranges (eg 10 °C - 26 °C) were included to validate methods and, as previously published experiments were conducted 40 years ago, to investigate likely

parasite adaptation.

Several constant temperatures were chosen for study under laboratory conditions to ensure that the methods employed in this work were consistent with the published data on the development of the free-living stages of *F. hepatica*. The variable temperatures that the UK isolate of *F. hepatica* eggs were exposed to were a gradual increase and decrease in temperature over the day as opposed to the rapid temperature variation studies conducteds by Claxton et al. [1999]. The published data are inconsistent with respect to the effect of freezing temperatures on the survival of *F. hepatica* eggs in the laboratory and the lower limit for survival and subsequent development. Over winter survival of parasites plays an important role in sverdispersionpring infections, it is important to be able to determine where parasite will survive the winter conditions.

The survival of metacercariae from different geographic locations was investigated, to determine whether there is evidence of regional adaptation to environmental conditions. The relationship between water availability and metacercarial survival is poorly understood. It is important to understand and to quantify the effect of desiccation on survival at different temperatures to investigate whether there is an interaction between the two climate variables.

2.2 Materials and Methods

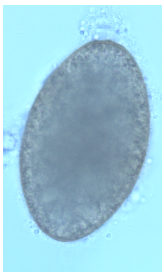
2.2.1 Acquisition of *F. hepatica* eggs for experimental work

F. hepatica eggs were obtained from fresh lamb livers and gall bladders collected at the slaughter line from an abattoir on Anglesey, immediately after the livers had been condemned by the meat inspector due to fluke damage. The livers and gall bladders were dissected to locate any adult flukes present, which were then incubated in phosphate buffered saline (PBS, pH 7.2) in 12-well ELISA plates at 37 °C overnight in order to stimulate egg shedding. The bile gathered from the livers and PBS containing the adult fluke was washed through three stacked sieves of decreasing mesh sizes (500μm, 150μm and 37μm) with the purpose of separating any debris present from the *F. hepatica*. All materials in the two larger sieves were discarded and *F. hepatica* eggs were collected from the 37μm mesh sieve and stored in distilled water at 4 °C. The water was changed weekly to prevent deterioration of the eggs. A stereo microscope was used to assess the proportion of damaged eggs or empty egg shells, 400 eggs were counted and examined this was repeated three times.

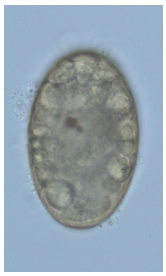
All of the experiments started with the same protocol for initial incubation. 100,000 eggs were incubated at each temperature in 60ml flat bottomed jars with 40ml of distilled water. These were then placed on the middle shelf of each incubator (Sanyo MIR-154-PE). Tinytag talk 2 temperature data loggers (Gemini Data Loggers Ltd (TK - 4023) Chichester) were used to monitor the temperature inside the incubator. Data loggers were set to record the temperature every 30 minutes. Distilled water was added to the jars during the experiment if more than 20ml had evaporated.

Before each egg experiment, a sample of fresh eggs was examined for empty shells, this

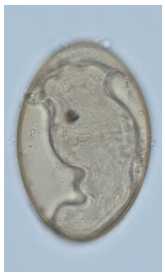
Figure 2.1: Development of *F. hepatica* eggs



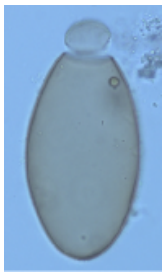
(a) Undeveloped egg



(b) Partially developed egg



(c) Fully developed egg



(d) Empty Shell

number was then deducted from the final count of eggs. Eggs were identified as either fully developed or not fully developed. Fully developed eggs contained a miracidium with a visible eye spot (Figure 2.1c) or an undamaged empty shell with the operculum open or missing (Figure 2.1d). Non fully developed eggs included undeveloped eggs and partly developed eggs (Figure 2.1a and 2.1b).

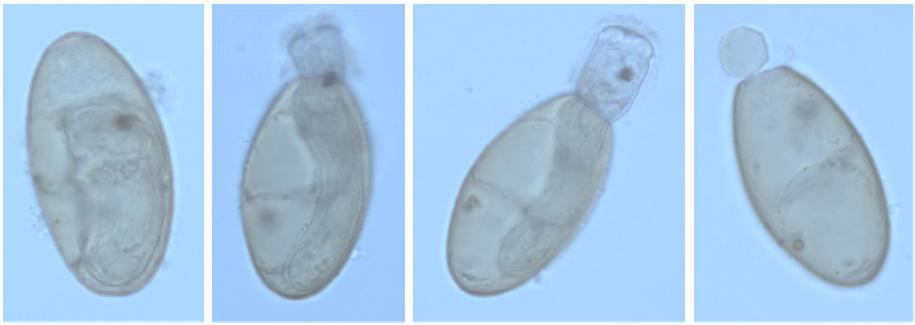


Figure 2.2: Fully developed miracidia hatching

2.2.2 The development and survival of *F. hepatica* eggs under different temperature conditions

Four experiments were conducted on *F. hepatica* eggs under temperature controlled conditions within the laboratory.

Experiment 1: Three sets of approximately 100,000 *F. hepatica* eggs were incubated at constant temperatures (18 °C, 22 °C and 26 °C) and one set at temperatures fluctuating between 16 °C and 28 °C with a 2 °C temperature change every 2 hours. At set time points during the experiment three samples of 400 eggs (1200 total eggs) were examined for signs of development per temperature condition.

Experiment 2: One set of *F. hepatica* eggs was refrigerated at 4 °C for 9 months in distilled water which was changed on a weekly basis. These eggs were incubated at 26 °C for 21 days using the same protocol described above. The eggs were examined for development using 3 replicates of 400 eggs.

Experiment 3: One set of *F. hepatica* eggs were also incubated at 40 °C for 1, 3 and 5 hours before being put into an incubator set to 26 °C for 21 days before being examined for development. Three replicates of 400 eggs were examined.

Experiment 4: Eight sets of *F. hepatica* eggs were exposed to temperatures of 4 °C, 0 °C, -2 °C, -4 °C, -5 °C, -6 °C, -7 °C and -8 °C, and samples were taken at 2, 4, 6, 8 and 14 days to be incubated at 26 °C for 21 days to assess viability according to the above protocol.

Mortality of *F. hepatica* eggs subjected to winter conditions

To validate whether *F. hepatica* eggs had the potential to survive over winter at pasture, fresh unembryonated eggs were partially buried in a field in Falcon tubes during the coldest months of the year (December to February). Three types of Falcon tube were used: 1) Falcon tubes with a small hole just below the cap and filled with 40ml of distilled water with the cap on; 2) Falcon tubes with a small hole below the cap and filled with soil and the cap on; 3) the bottoms of the Falcon tube removed and a permeable silk membrane secured over the end, the Falcon tube was then filled with soil with no cap to allow the free flow of water. The soil used was autoclaved commercial top soil (Verve Top Soil 25 L, Product code:04119906) which was passed through a 150μm sieve to remove small soil particles. The Falcon tubes were half buried in the soil to ensure that they remained upright. Two data loggers were used to monitor the temperature of the soil (Tinytag Plus 2 - TGP-4020) and the humidity at grass root level (Tinytag Plus 2 TGP-4500).

Eight of each type of Falcon tube containing approximately 100,00 eggs were kept in situ for up to 58 days. After week 3, 4, 5, 6, 7 and 8 of the experiment, one of each type of Falcon tubes was removed from the field and eggs that had been kept in soil separated from it by flushing water through 500μm, 150μm, 37μm sieves, with *F. hepatica* eggs being retained on the 37μm sieve. The eggs were then transferred into water was decanted into fresh pots and the water replaced with fresh distilled water. The eggs were then washed out of the 37μm sieve and sedimentation performed several times with distilled water to remove most soil particles. Eggs were then incubated at 26 °C for 21 days, after which their mortality was assessed. The experimental design and set-up were conducted by the author. The assessment of mortality of the eggs was done by a 3rd year veterinary student

Figure 2.3: Forms of Falcon tubes used for field experiments

(a) Filled with water and capped

(b) Filled with soil and bottom drain and no cap

(c) Filled with soil and no bottom drain with cap

from the University of Liverpool, trained and supervised by the author.

A control sample of eggs, that were not exposed to field conditions, were incubated in water kept at 26 °C at the start of the experiment. The three replicates of 400 control eggs were examined after 21 days incubation and had a mean mortality of 34% (SD 0.88) (Table 2.9). For the field samples, at each time point, three replicates of approximately 400 eggs were examined, (Table 2.9). Over the study period during the winter of 2012-13, temperatures experienced by the eggs were monitored using a tinytag data logger and ranged between -2 °C and 25 °C (Figure 2.18).

The effect of temperature and moisture on metacercarial survival conducted at Ridgeway Research

Two experiments were conducted on the *F. hepatica* metacercariae at Ridgeway Research. These Experiments which involved passaging the parasite through the intermediate host, *G. truncatula*, were carried out at Ridgeway Research in South Wales. The experiments conducted by Ridgeway Research were done on two isolates of *F. hepatica*; a Welsh resistant isolate maintained at Ridgeway Research and an isolate from Italy isolated from a naturally infected sheep (Dr Laura Rinaldi, University of Naples, personal communication).

Undeveloped *F. hepatica* eggs obtained from Italian sheep were transported in a chilled container to Ridgeway Research. The eggs were incubated at 26 °C for 14 days and hatched miracidia then exposed to a naive colony of *G. truncatula*. The snails were kept at room temperature and fed on sterile algae produced on-site until the parasite was fully developed. The snails were induced to shed metacercariae by reducing the temperature to 10 °C and then returning them to room temperature. The snails were placed into individual wells surrounded by parafilm upon which the metacercariae then became encysted. The metacercariae were refrigerated at 4 °C until the start of the experiments.

Experiment 1 was based on that described by Boray and Enigk [1964], using metacercariae from Wales and Italy to examine variations in the response of isolates obtained from two geographically different regions. Metacercariae were fully immersed in distilled water throughout the experiment and incubated at 15 °C, 20 °C, 25 °C and 30 °C, plus a control at 5 °C. Repeated measurements of three replicates of approximately 100 metacercariae were assessed for viability at the start and then every 7 days for 6 weeks. Viability was determined by visual examination of the metacercariae under 10x magnification without

excystment. Ridgeway Research conducted a small pilot study to determine if the viability of the metacercariae could be determined with the parasite still within its cyst as this allowed for repeated measures to be taken. The metacercariae were examined for two defined kidney shaped bodies inside the cyst called flame cells. If these cells were visible then the metacercariae were considered potentially infective. The Welsh isolates acted as a control to compare the results with other experimental metacercariae work conducted in the United Kingdom.

Experiment 2 examined the effect of dehydration on the metacercariae. The metacercariae were visually examined using the methods described above. For this experiment, the temperature was kept at a constant 20 °C but rather than being fully submerged in water, the metacercariae were divided into 4 categories. The control group was fully submerged in water, the other groups had 5ml added every 2, 5 and every 7 days, and in one group, no water was added during the experiment (Table 2.1). Samples were kept in petri dishes inside an incubator so that the water could evaporate, a bowl of water was kept in the incubator to ensure that the relative humidity remained high. The metacercariae were examined weekly for 7 weeks to determine their viability.

Table 2.1: The moisture conditions metacercariae were exposed to during experiments to assess the effects of dehydration

Metacercariae condition	Time
Controls in water	49 days
Watered every other day	49 days
Watered every five days	49 days
Watered every seven days	49 days
Dry, never watered	49 days

Metacercarial survival under two moisture condition and various constant temperatures

The metacercariae were stored in distilled water at 4 °C in darkness with the water changed once a week until the start of the experiment, then divided into groups of approximately 100 cysts (actual numbers ranging from 55 to 127) using a scalpel to cut the parafilm that the metacercariae had encysted on. Three sets of 100 metacercariae were used for each temperature and moisture condition. Metacercarial survival at 10 °C, 15 °C, 20 °C and 25 °C under two water withholding conditions was investigated using a UK isolate of *F. hepatica* obtained from Dr Jane Hodgkinson (Veterinary Parasitology, University of Liverpool). Metacercariae were either fully immersed in water, or 1ml of water was added to the dry metacercariae every two days. The metacercariae immersed in water were placed into a covered petri dish to avoid evaporation. The metacercariae exposed to drying conditions were put into an uncovered petri dish so the water could evaporate. Every two days, 1ml of water was pipetted onto the parafilm containing the metacercariae in the petri dishes. The parafilm containing the metacercariae completely dried out between the additions of water. Adding water to the parafilm allowed it to be flattened onto a slide and viewed under a microscope. A bowl of water was also placed inside the incubator to keep the humidity above 90%, as these conditions reflect those of the herbages. The samples were kept out of the incubator for no more than 30 minutes at any time point. Tinytag plus 2 temperature and humidity data loggers were put into each incubator, measuring the temperature every 5 minutes.

Metacercariae were examined every other day for the first 12 days, after which they were counted every four days until the end of the experiment. They were examined using 10x magnification on a compound microscope. Metacercariae were considered alive if the

flame cells were visible [Sasithorn, 2003]. This assessment was made without excysting the metacercariae and repeated counts were conducted on the same metacercariae. The total number of metacercariae in each sample where the flame cells were no longer visible (i.e. considered to be dead) were counted at each time point.

2.2.3 Statistical analysis

The time taken for 50% of the viable *F. hepatica* eggs to develop to the point of hatching was determined using a three parameter logistic (sigmoid) curve (Equation 2.1). It was fitted to the temperature data between 14 °C and 26 °C. Parameter values were estimated by fitting the best curve by the method of least squares. The Excel package 'solver' was used to calculate the parameter values that gave the best least squares fit. Logistic models were to be fitted to data, as the proportion of eggs developing shows an initial steady increase followed by a plateau at the maximum percentage of eggs that will develop. The time taken for 50% of development to occur was considered as the mid point of the sigmoid curve.

$$P_t = \frac{L}{(1 + \exp(-K \times (t - X0)))} \tag{2.1}$$

Where P_t = Proportion of eggs that have developed by time t, L = Proportion of viable eggs which developed (maximum value), K = steepness of curve, X0 = sigmoid mid-point and t = time.

2.3 Results

2.3.1 Development of *F. hepatica* eggs held at constant and varying temperatures

Table 2.2 summarises the parameters of the logistic curves that were estimated from the data from different temperature conditions. The maximum predicted percentage of eggs developing from the logistic model was similar in those experiments where it was possible to estimate this parameter, varying from 35% to 44%. Thus, over 50% of eggs, even under controlled experimental conditions, did not develop (Table 2.3). At the highest temperature studied (26 °C), half of the viable eggs had developed by 12 days. At lower temperatures, the time to this point progressively increased, 19 days at 22 °C and 33 days at 18 °C and beyond 40 days at 14 °C. For temperatures varying between 16 °C and 28 °C, the development of eggs over time was similar to that of eggs held at a constant temperature of 22 °C (which was the mean temperature for variation between 16 and 28 °C).

Table 2.2: Logistic regression for the development time of *F. hepatica* eggs held at constant and varying temperatures

Temperature	Estimated time (Days) by which 50% of viable eggs had developed	Estimated percentage of viable eggs (%)	Steepness of curve
26 °C	12.31	35.05	2.05
22 °C	18.83	44.45	0.52
18 °C	32.51	40.89	0.20
16 °C - 28 °C	17.90	43.49	0.97

Table 2.3: Percentage of *F. hepatica* eggs that developed when kept at constant or varying temperatures

Time (Day)	26 °C Mean % (SD)	22 °C Mean % (SD)	16-28 °C Mean % (SD)	18 °C Mean % (SD)
10	0 (0)	0 (0)	0 (0)	0 (0)
11	6.28 (2.69)	-	-	-
12	10.09 (0.99)	-	0 (0)	-
13	29.33 (3.72)	0 (0)	0.77 (0.76)	-
14	35.51 (5.35)	1.14 (0.60)	1.11 (1.06)	-
15	32.99 (4.51)	1.71 (0.94)	1.64 (0.86)	-
16	29.49 (3.15)	9.38 (2.72)	-	-
19	41.40 (3.70)	28.96 (3.13)	23.14 (2.54)	-
21	22.67 (3.50)	28.23 (5.45)	38.21 (6.57)	-
23	38.95 (3.57)	40.00 (7.25)	42.17 (9.47)	2.64 (1.68)
24	-	-	42.80 (3.45)	4.34 (2.38)
25	30.15 (3.11)	47.31 (8.66)	-	11.02 (1.57)
26	-	-	43.36 (3.85)	13.12 (3.28)
27	34.08 (1.84)	32.48 (4.02)	-	7.68 (1.34)
28	40.00 (4.27)	-	-	-
30	-	38.72 (7.32)	40.85 (5.05)	15.66 (2.84)
35	49.08 (2.27)	56.07 (6.45)	-	24.00 (0.50)
39	-	55.87 (0.76)	50.08 (3.33)	32.60 (7.58)

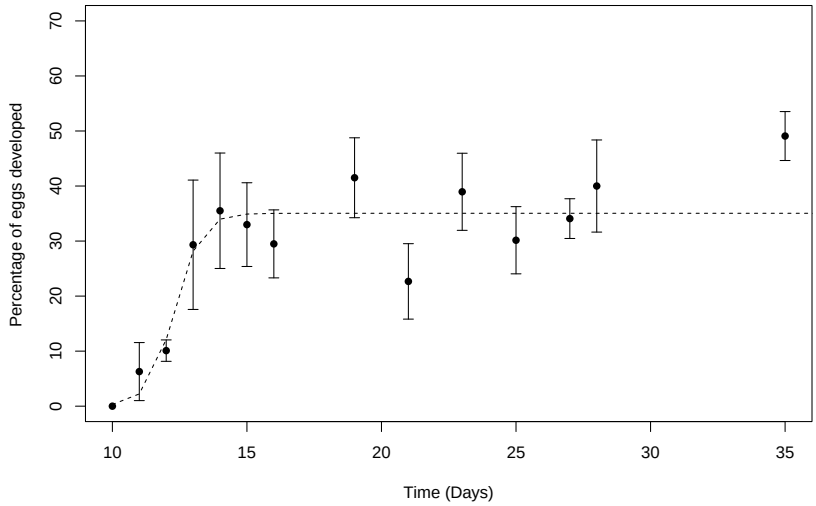


Figure 2.4: Development of *F. hepatica* eggs held at 26 °C with 95% confidence intervals.

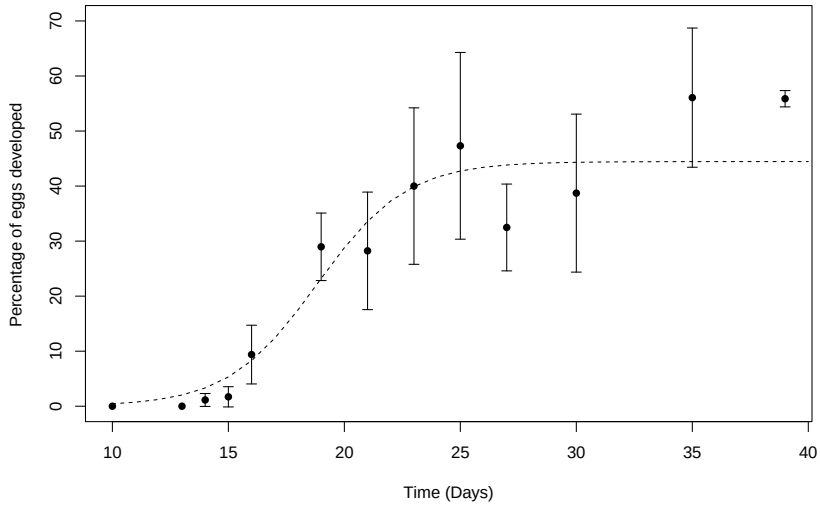


Figure 2.5: Development of *F. hepatica* eggs held at 22 °C with 95% confidence intervals.

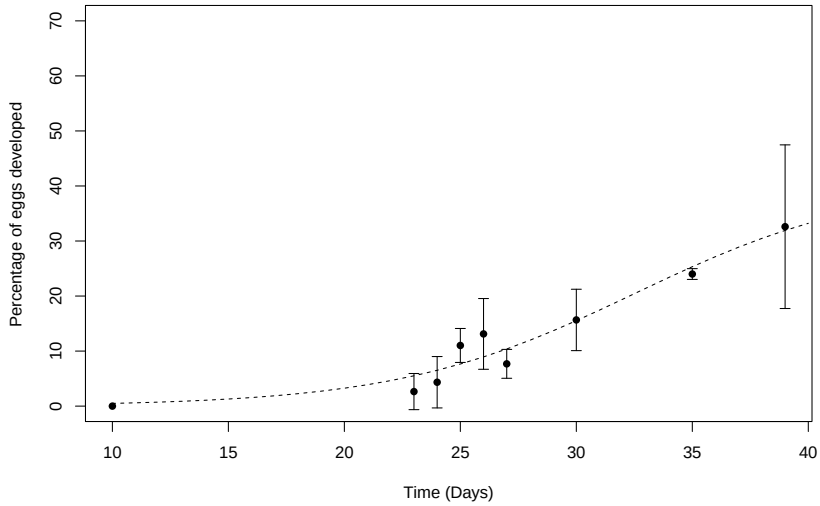


Figure 2.6: Development of *F. hepatica* held at 18 °C with 95% confidence intervals.

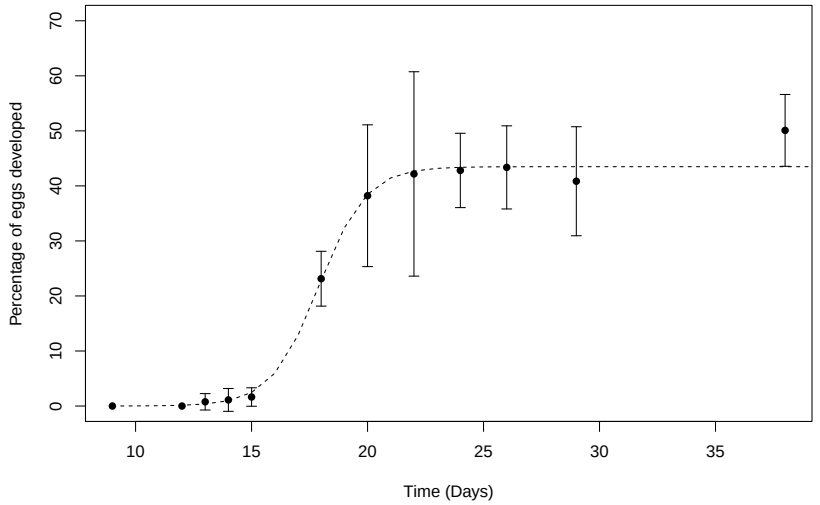


Figure 2.7: Development of *F. hepatica* eggs held at varying temperatures between 16 and 28 °C over 12 hours with 2 °C temperature changes every 2 hours with 95% confidence intervals.

Figure 2.8 shows the published data on the effect of temperature on the development time of *F. hepatica* eggs held at constant temperatures (Black points) [Roberts, 1950, Kendall, 1953, Al-Habbib, 1974, Nice, 1979] and the data gathered as part of this thesis at constant (red cross) and varying (blue plus) temperatures. In the temperature conditions studied by the author, 26 °C was the temperature at which most rapid development was observed, with the estimated time to 50% development at 12 days. This time increased by more than 2.5 times at 18 °C to 33 days.

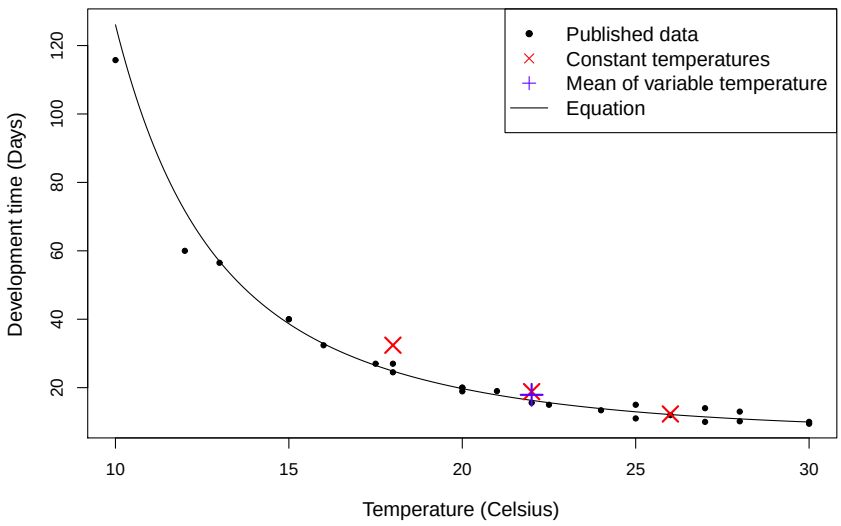


Figure 2.8: Estimated time to 50% development of *F. hepatica* eggs under laboratory conditions, combining thesis data together with published data from Roberts [1950], Kendall [1953], Al-Habbib [1974], Nice [1979].

2.3.2 Mortality of *F. hepatica* eggs exposed to low temperatures, below 5 °C

The controls, which were not kept at low temperatures, showed a mean mortality of 32.83% (S.D. of 6.79) after 21 days incubation at 26 °C (Table 2.4). The mean egg mortality for eggs held at different temperatures for different periods of time is shown in Table 2.4. Figure 2.9 shows the mortality of eggs held at 4 °C for different periods of time. The data points were created using six replicates of eggs, the graph also contains a the best fitting linear regression line. The regression coefficient for change in mortality with time is shown in Table 2.5 and indicates that there was no significant change in mortality compared to the control group at any duration of being held at 4 °C up to 14 days. In the case of eggs kept at 4 °C for 9 months then incubated at 26 °C there was 68.30% (SD 1.71) mortality of the eggs. In the case of eggs that hatched, the resultant miracidia appeared active and healthy, swimming rapidly and with directionality.

The graphs for the results at the lower temperatures are shown in Figures 2.10 to 2.16 and the corresponding regression coefficients are shown in Tables 2.5 and 2.6. For temperatures of 0 °C, there was no strong statistical evidence of a correlation between time spent at 0 °C for up to 14 days and increased mortality (Table 2.5). This demonstrates that unembryonated *F. hepatica* eggs can survive freezing temperatures without increased mortality. Increased mortality was observed at temperatures between -2 °C and -8 °C and there was a positive correlation with time spent at low temperatures and increased egg mortality. The highest increase in mortality with time was observed in eggs kept at -8 °C, with 100% mortality after 2 days exposure (Figure 2.16). A small proportion of *F. hepatica* eggs hatched after 2-8 days at -7 °C although 100% mortality was observed after 14 days.

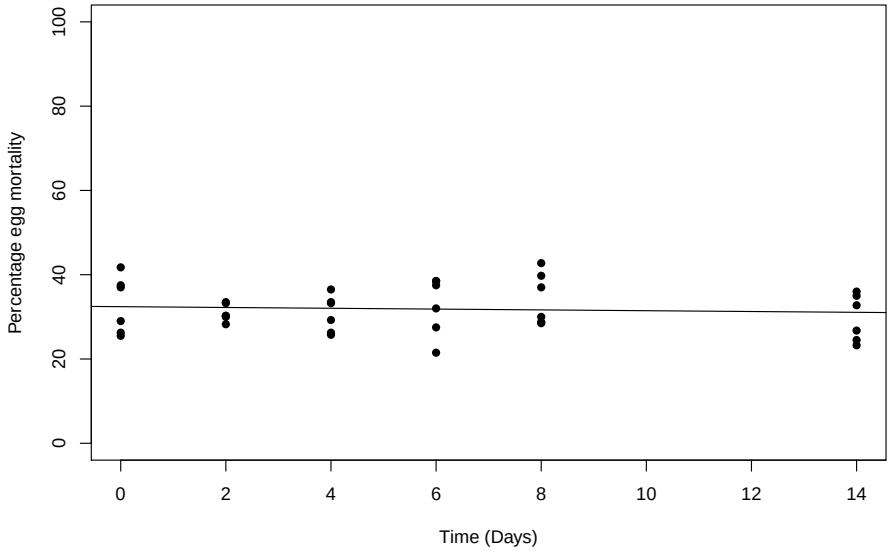


Figure 2.9: Mortality of *F. hepatica* eggs held at 4 °C for 14 days

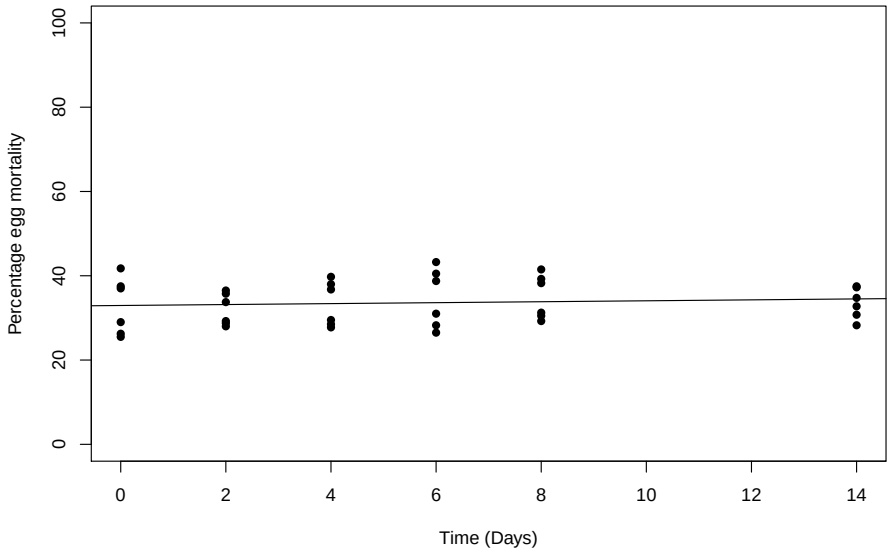


Figure 2.10: Mortality of *F. hepatica* eggs held at 0 °C for 14 days

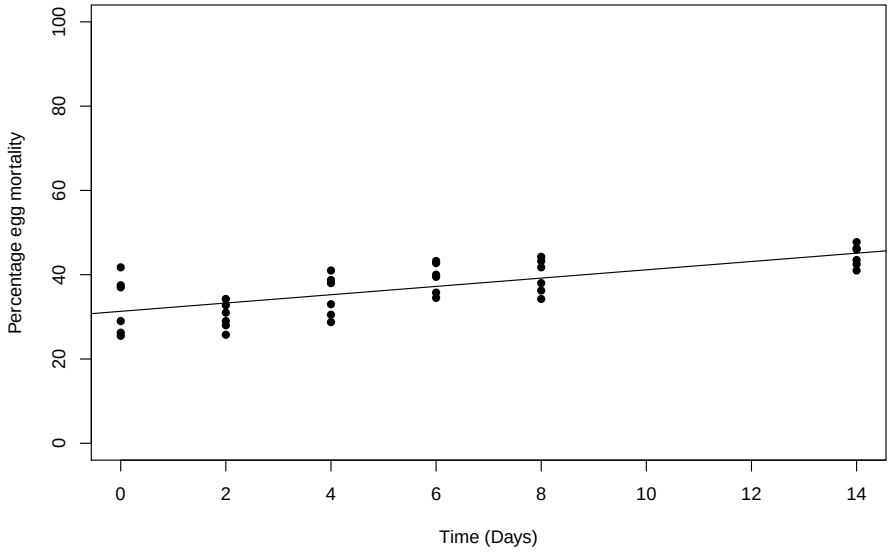


Figure 2.11: Mortality of *F. hepatica* eggs held at -2 °C for 14 days

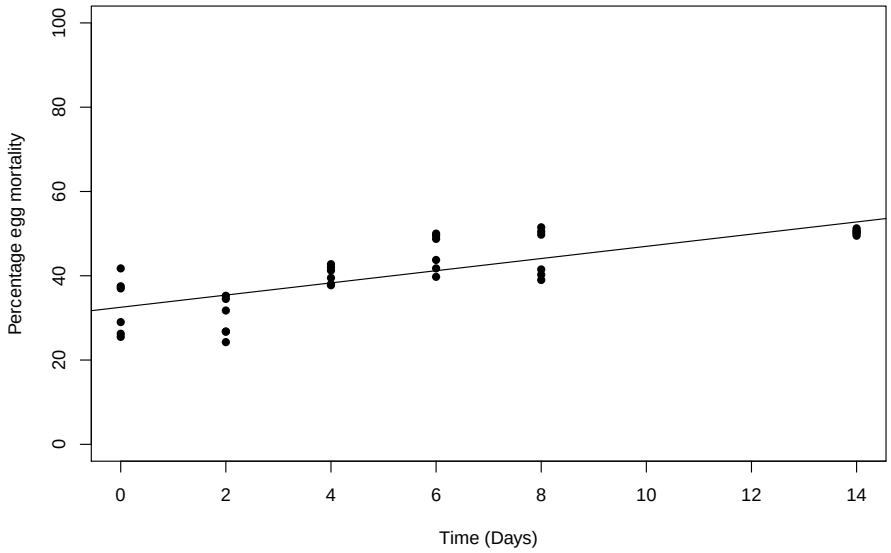


Figure 2.12: Mortality of *F. hepatica* eggs held at -4 °C for 14 days

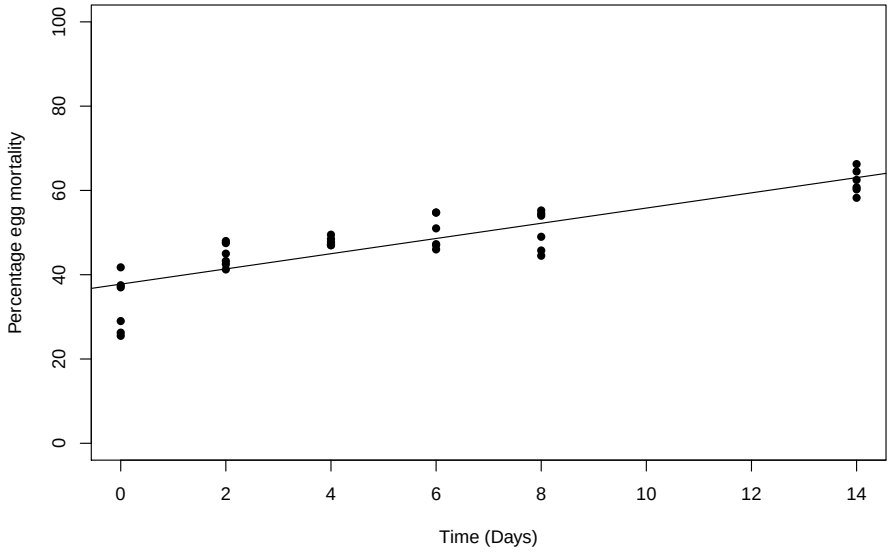


Figure 2.13: Mortality of *F. hepatica* eggs held at -5 °C for 14 days

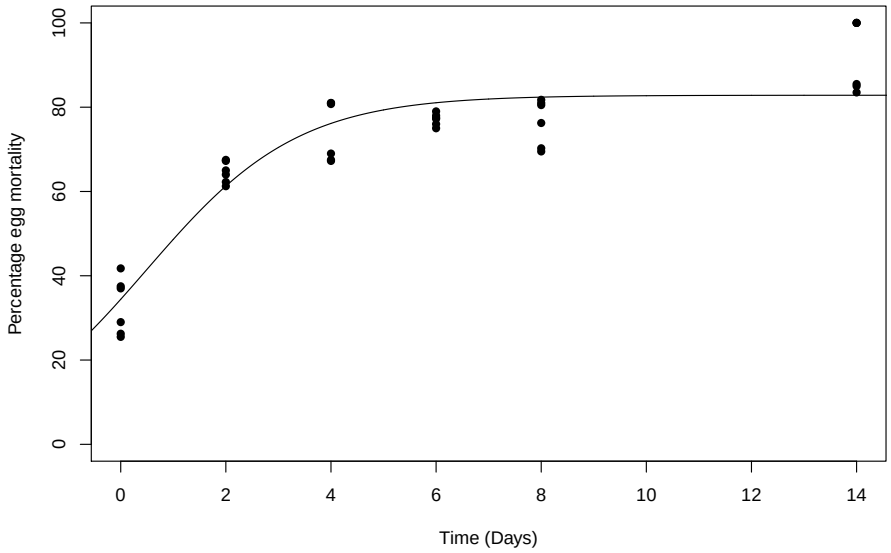


Figure 2.14: Mortality of *F. hepatica* eggs held at -6 °C for 14 days

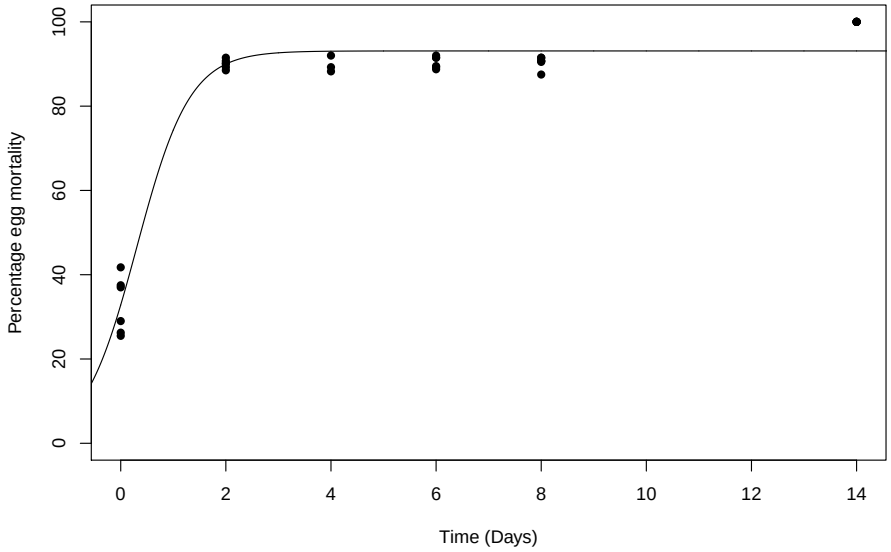


Figure 2.15: Mortality of *F. hepatica* eggs held at -7 °C for 14 days

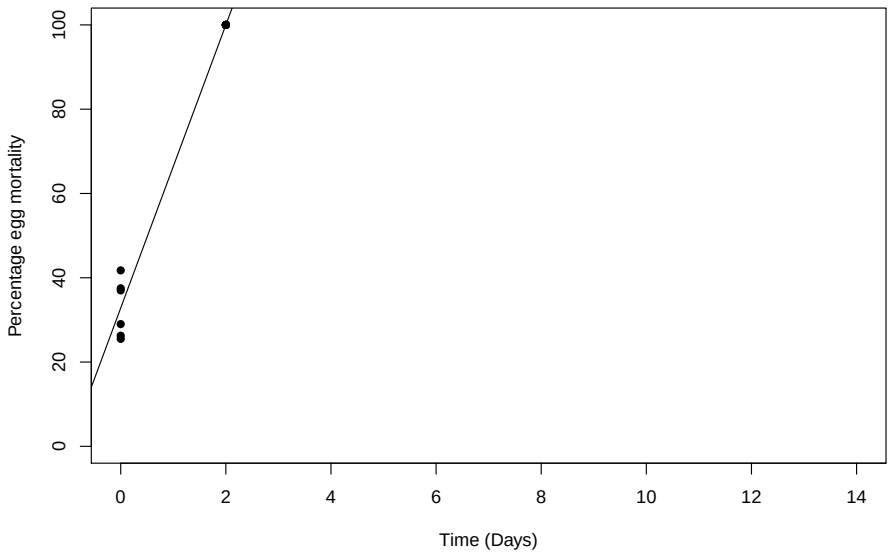


Figure 2.16: Mortality of *F. hepatica* eggs held at -8 °C for 2 days

Table 2.4: Egg mortality (%) after exposure to cold temperatures controls were incubated at 26 °C (based upon three replicates of 400 eggs in each of two different samples, i.e. six replicates in all.)

Temperature	2 days Mean % (SD)	4 days Mean % (SD)	6 days Mean % (SD)	8 days Mean % (SD)	14 days Mean % (SD)
4 °C	30.91 (2.05)	30.75 (4.34)	32.58 (6.97)	34.46 (6.18)	29.71 (5.56)
0 °C	32.00 (3.78)	33.38 (5.36)	34.71 (7.01)	35.00 (5.26)	33.54 (3.67)
-2 °C	30.13 (3.15)	35.00 (4.95)	39.29 (3.57)	39.63 (4.05)	44.50 (2.57)
-4 °C	29.88 (4.58)	40.21 (2.11)	45.58 (4.40)	45.42 (5.74)	50.38 (0.61)
-5 °C	44.58 (2.74)	47.83 (1.00)	50.13 (3.97)	50.50 (4.72)	62.33 (2.94)
-6 °C	64.54 (2.56)	74.42 (7.15)	77.13 (1.43)	76.54 (5.51)	92.33 (8.42)
-7 °C	90.04 (1.07)	89.83 (1.94)	90.42 (1.40)	90.42 (1.49)	100 (0)
-8 °C	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)
Controls (26 °C)	32.83 (6.79)				

Table 2.5: Linear regression results for *F. hepatica* eggs held at low temperatures

Temperature	Degrees of Freedom	Regression Coefficient (slope)	SE	p-value
4 °C	34	-0.096	0.201	0.636
0 °C	34	0.111	0.192	0.565
-2 °C	34	0.986	0.165	<0.001
-4 °C	34	1.446	0.198	<0.001
-5 °C	34	1.805	0.178	<0.001
-8 °C	10	33.583	1.386	<0.001

Table 2.6: Logistic regression for *F. hepatica* eggs held at low temperatures

Temperature	Estimated time of 50% mortality (Days)	Estimated percentage egg mortality (%)	Steepness of curve
-6 °C	0.69	82.8	0.49
-7 °C	9.98	93.1	0.3

2.3.3 Mortality of *F. hepatica* eggs exposed to high temperature 40 °C

Eggs were exposed to 40 °C for between 1 and 5 hours then moved to 26 °C to develop for 21 days. Controls were incubated at 26 °C for 21 days. The mortality of the control eggs was 47% (SD 2.99) (Figure 2.17). After 1 hour at 40 °C, the mortality remained similar to the control (mean 47%, SD 6.23), however, after 5 hours, this rose to 61% (SD 2.77). There was a positive correlation between time spent at 40 °C and egg mortality Figure 2.17 and Table 2.8.

Table 2.7: Average mortality of *F.hepatica* eggs exposed to 40 °C

Time spent at 40 °C (Hours)	Mean Mortality (%) (SD)
0	47.37 (2.99)
1	47.63 (6.23)
3	57.52 (0.52)
5	60.64 (2.77)

Table 2.8: Regression Correlation for *F. hepatica* eggs kept at 40 °C with 95% confidence intervals

Temperature	Degrees of Freedom	Regression Correlation (Slope)	Standard Error	p-value
40 °C	10	2.97	0.5594	<0.001

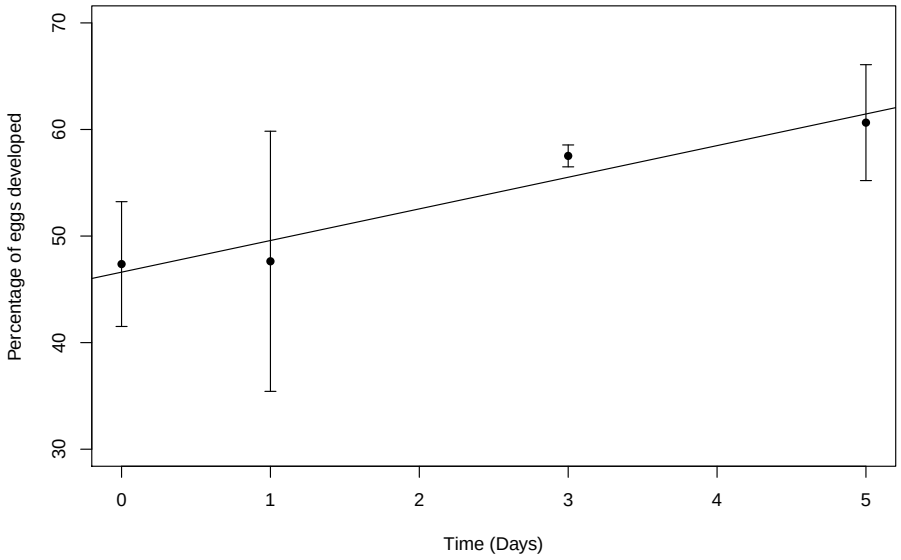


Figure 2.17: Mean mortality of *F. hepatica* held at 40 °C for 1, 3 and 5 hours

2.3.4 Mortality of *F. hepatica* eggs under winter conditions

The eggs kept outside in water (Figure 2.19) showed an increase in mortality over time, although the association was weak, with a regression coefficient of 0.15 (p-value = 0.06). However, if the apparently anomalous data at 22 days are excluded, then the association becomes non-significant at the 5% level (p-value = 0.1). Eggs kept in soil (Figures 2.20 and 2.21) showed a difference in mortality from controls after up to 58 days in the field. However, the observations at 22 days also appear to be anomalous as was seen in Figure 2.19.

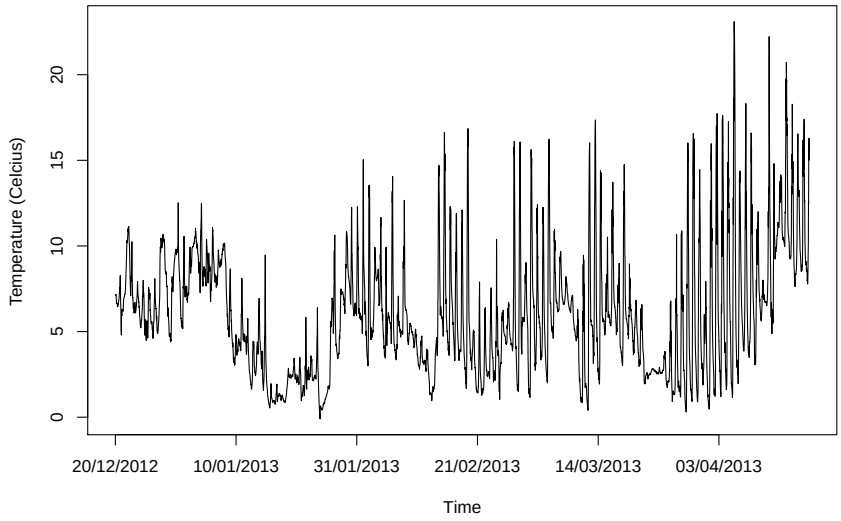


Figure 2.18: Temperature recorded by data logger during the study period (Winter 2012-13)

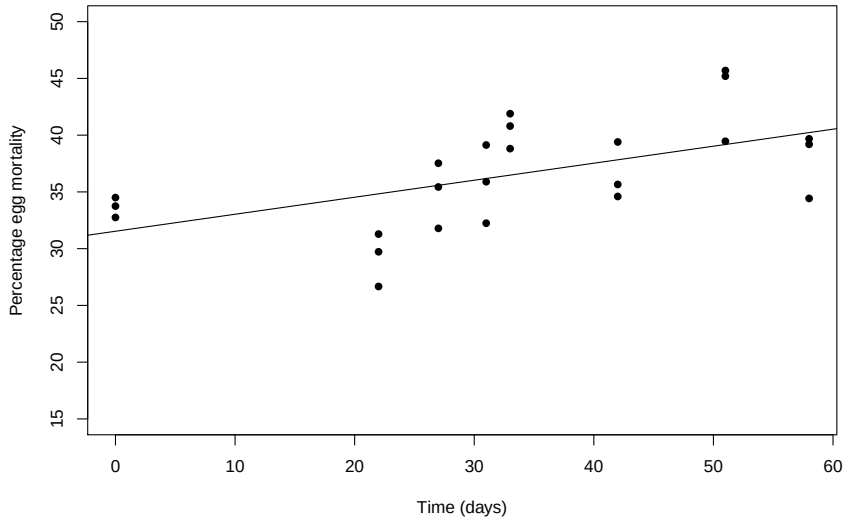


Figure 2.19: Mortality of *F. hepatica* eggs in the environment in Falcon tubes in water over winter

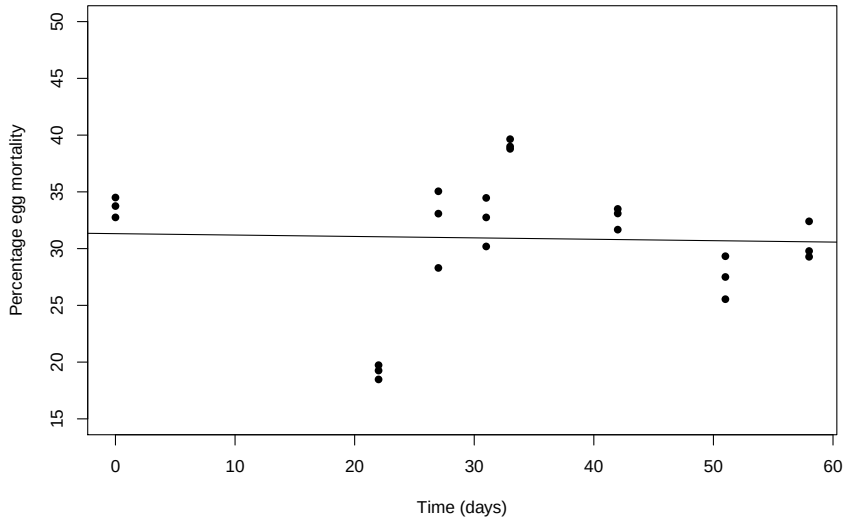


Figure 2.20: Mortality of *F. hepatica* eggs in the environment in Falcon tubes filled with soil with a permeable membrane at the bottom

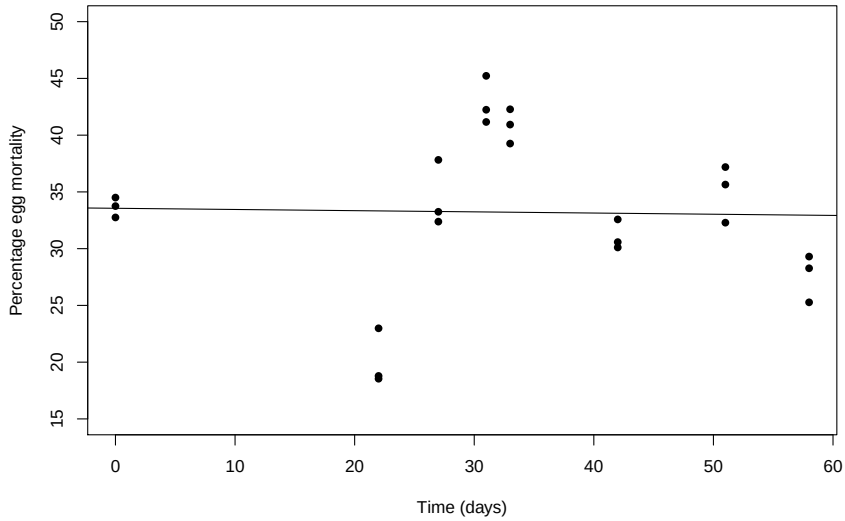


Figure 2.21: Mortality of *F. hepatica* eggs in the environment in sealed Falcon tubes with soil with no draining

Table 2.9: Mean percentage mortality of *F. hepatica* eggs exposed to winter conditions in Falcon tubes

Time in field	Tubes filled with water Mean % (SD)	Tubes filled with soil and permeable membrane at bottom Mean % (SD)	Tubes filled with soil with solid bottom Mean % (SD)
Control	33.67 (0.88)	33.67 (0.88)	33.67 (0.88)
22	29.23 (2.35)	19.15 (0.63)	20.10 (2.50)
27	34.92 (2.91)	32.15 (3.47)	34.48 (2.92)
31	35.76 (3.45)	32.47 (2.15)	42.88 (2.11)
33	40.50 (1.56)	39.14 (0.45)	40.82 (1.51)
42	36.55 (2.52)	32.75 (0.96)	31.08 (1.31)
51	43.45 (3.46)	27.46 (1.89)	35.04 (2.50)
58	37.77 (2.90)	30.49 (1.68)	27.62 (2.09)

Table 2.10: Regression coefficients for *F. hepatica* eggs kept in Falcon tubes over winter

Condition	Degrees of Freedom	Regression Coefficients	Standard Error	p-value
in water	22	0.15	0.048	0.06
In soil with drainage	22	-0.01	0.070	0.862
In soil with no drainage	22	-0.01	0.88	0.906

2.3.5 Survival of metacercariae from two geographically different areas kept at constant temperatures

Figure 2.22 shows the mortality of the metacercarial samples from Wales kept at these five temperatures for up to 49 days. The metacercariae kept at 5 °C, as expected, had the lowest mortality throughout the study period with 19% mortality after 49 days. The mortality rate increased with higher temperatures and in metacercariae kept at 30 °C, there was 100% mortality after 49 days. Figure 2.23 shows the results for the Italian metacercarial samples, which show a similar pattern of increasing mortality with time and temperature, although at each time, the mortality in these samples was higher than in the Welsh samples. There was 100% mortality at 42 days at 25 °C and after 35 days at 30 °C.

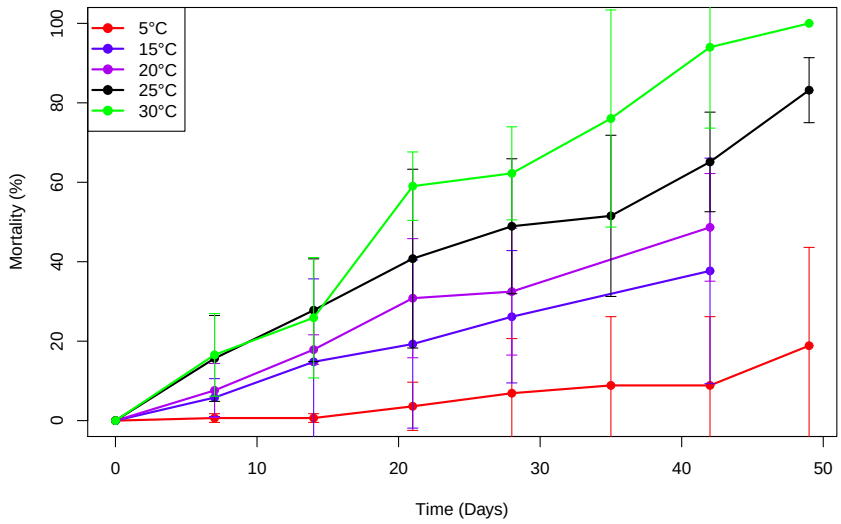


Figure 2.22: Mortality of Welsh metacercariae in water at temperatures between 5 °C and 30 °C (at 15 °C and 20 °C no samples were examined on days 35 and 49) with 95% confidence intervals

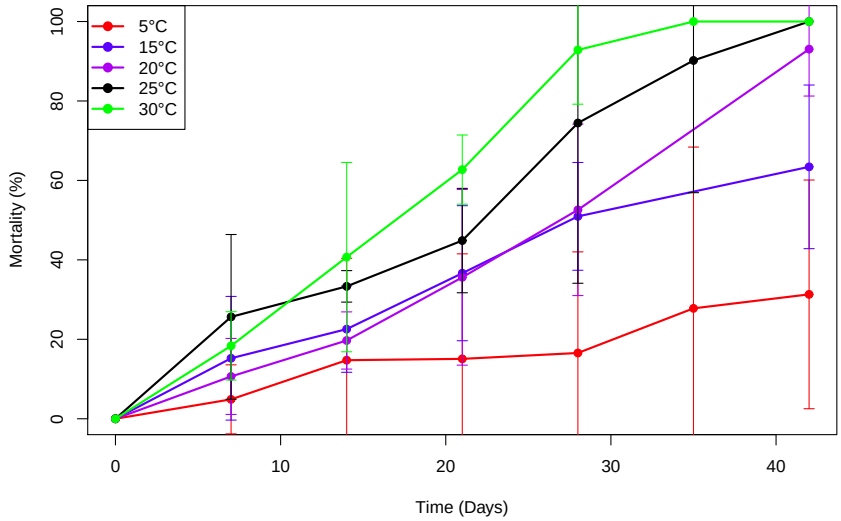


Figure 2.23: Mortality of Italian metacercariae in water at temperatures between 5 °C and 30 °C with 95% confidence intervals

Table 2.11 and Figure 2.24 show the estimated daily death rates (as a proportion) for the metacercarial samples kept at different temperatures. For example, at 5 °C, it was estimated that the daily death rate was 0.002 or 0.2% in the Welsh isolate. The daily death rates in the Welsh samples and the Italian samples increased with increasing temperature. The highest daily death rate was observed at 30 °C in both samples (Welsh 3.2% and Italian 5.9%). At each temperature, mortality rates were higher in the Italian samples apart from day 49 at 300 °C where both had 100% mortality.

Table 2.11: Estimated daily death rates of *F. hepatica* metacercariae in samples from Wales and Italy kept at different temperatures

Temperature	Daily death rate Welsh sample (95% CI)	Daily death rate Italian sample (95% CI)
5 °C	0.002 (0.001-0.003)	0.004 (0.001-0.007)
15 °C	0.005 (0.003-0.007)	0.011 (0.009-0.012)
20 °C	0.007 (0.006-0.008)	0.027 (0.016-0.037)
25 °C	0.014 (0.011-0.016)	0.040 (0.021-0.059)
30 °C	0.032 (0.021-0.043)	0.059 (0.043-0.075)

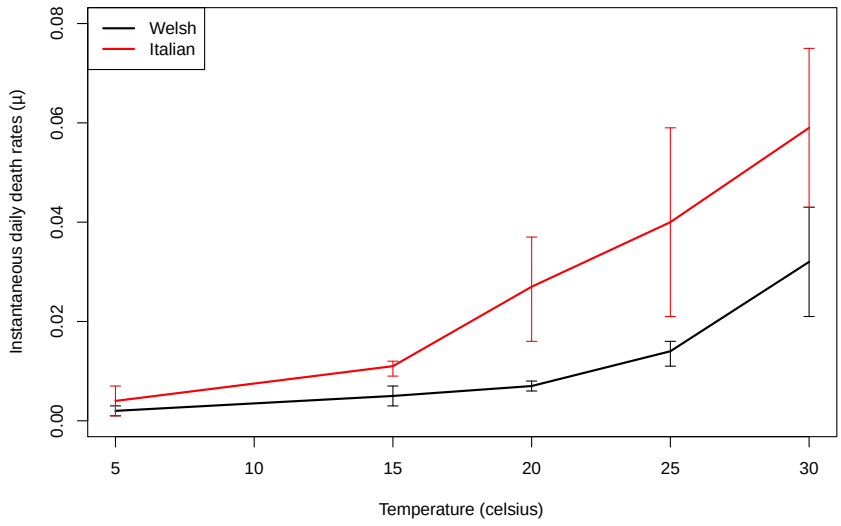


Figure 2.24: Estimated daily death rates of *F. hepatica* metacercariae in samples from Wales (black line) and Italy (red line) kept at different temperatures with 95% confidence intervals.

2.3.6 Survival of metacercariae from two geographically different areas kept at constant temperatures and with different levels of water available

Figure 2.25 shows the mortality of Welsh metacercariae at different times during the study period. Under all conditions, including controls, the mortality increased over the study period, and after day 43, all metacercariae exhibited over 80% mortality. Adding water every 2 days, and then at a decreased frequency up to 43 days, resulted in similar patterns in mortality of the metacercariae. Figure 2.26 shows the results for the Italian metacercariae. The controls had low mortality up to day 10, after which there was a steep rise until day 15. All of the metacercariae exposed to irregular water availability had similar mortality, substantially higher than that in controls. The estimated daily death rate of the Italian metacercariae was higher than for the Welsh samples under all moisture conditions (Table 2.12 and Figure 2.27).

Table 2.12: Estimated daily death rates of *F. hepatica* metacercariae in samples from Wales and Italy kept at 20 °C under different moisture conditions

Water treatment	Welsh isolate (95% CI) Mortality	Italian isolate (95% CI) Mortality
Control (in water)	0.010 (0.008-0.011)	0.030 (0.022-0.038)
Every other day	0.026 (0.020-0.033)	0.084 (0.055-0.113)
Every 5 days	0.025 (0.019-0.031)	0.084 (0.056-0.112)
Every 7 days	0.034 (0.026-0.043)	0.102 (0.081-0.122)
No water	0.036 (0.028-0.043)	0.103 (0.085-0.122)

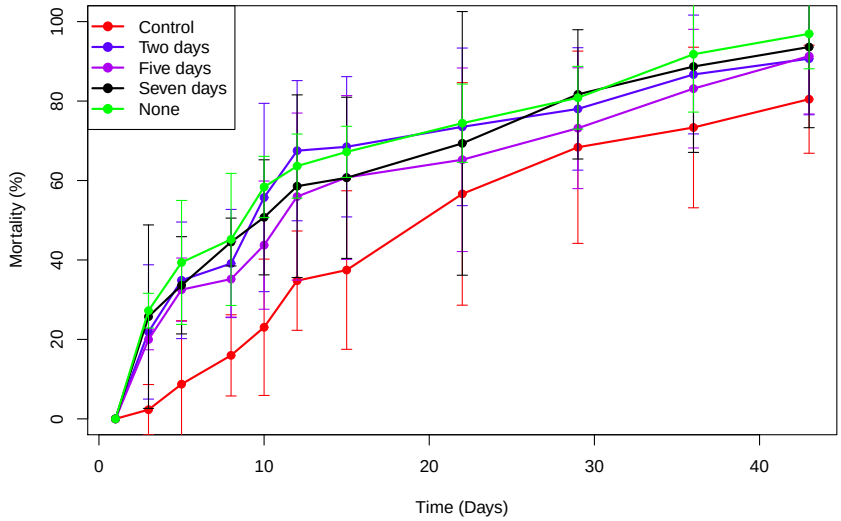


Figure 2.25: Mortality of Welsh metacercariae incubated at 20 °C under various conditions of water availability with 95% confidence intervals

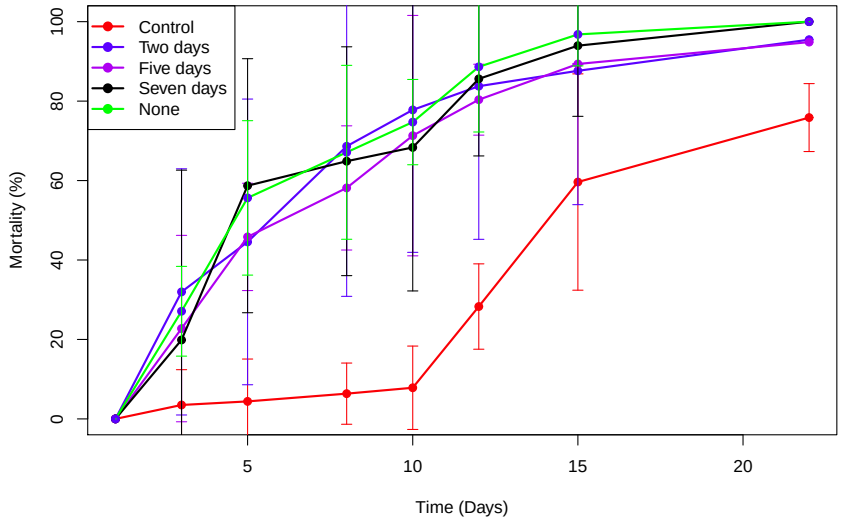


Figure 2.26: Mortality of Italian metacercariae incubated at 20 °C under various conditions of water availability with 95% confidence intervals

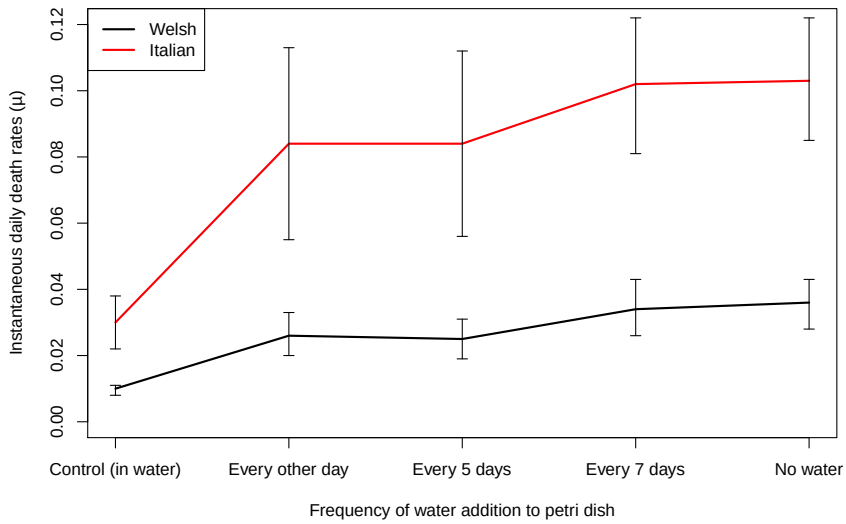


Figure 2.27: Estimated daily death rates of *F. hepatica* metacercariae in samples from Wales and Italy kept at 20 °C under different moisture conditions with 95% confidence intervals

2.3.7 Survival of metacercariae kept at constant temperatures under different conditions of water availability

Figure 2.28 shows the mean mortality of metacercariae from the UK incubated at constant temperatures in water. The lowest mortality was observed at 10 °C and the highest mortality 25 °C, in which there was 80% by day 52. The metacercariae with limited water availability (Figure 2.29) showed similar mortality rates to those in water up to 20 °C but at 25 °C the mortality of metacercariae was substantially higher, with 100% mortality observed after 19 days. The estimated daily death rates are shown in Table 2.13 and Figure 2.30.

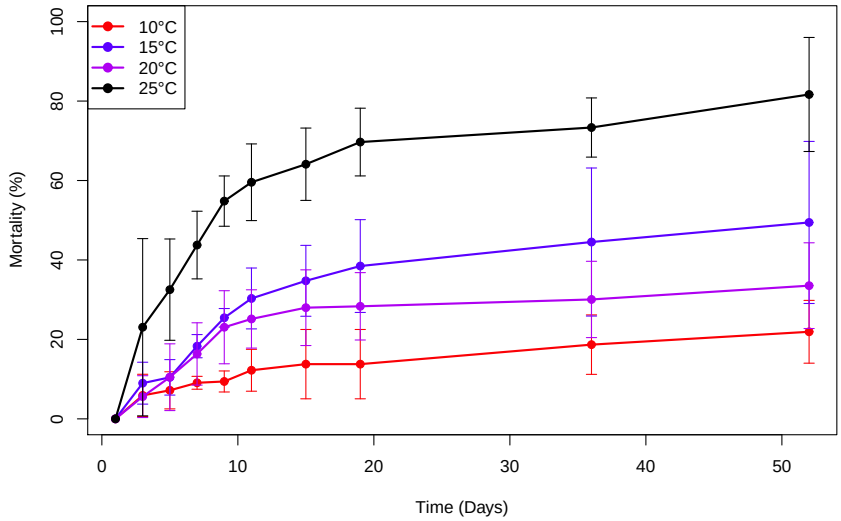


Figure 2.28: Mortality of a UK isolate of metacercariae kept in water between 10 °C and 25 °C with 95% confidence intervals

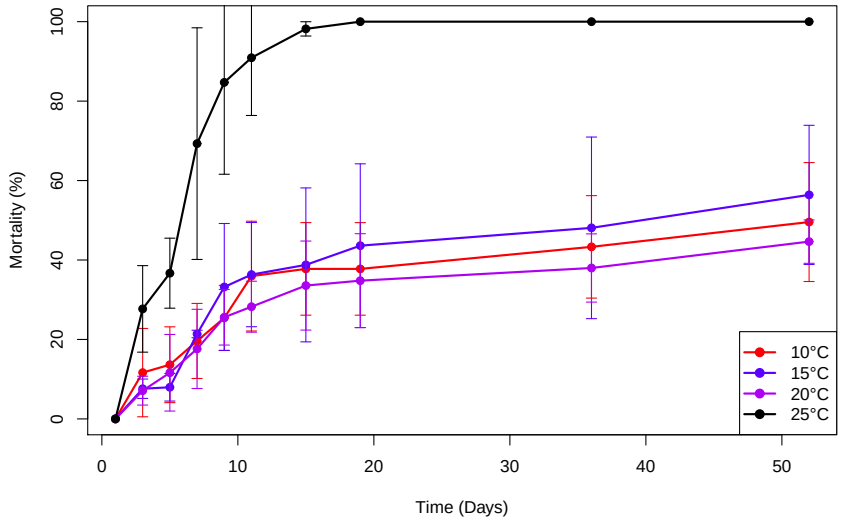


Figure 2.29: Mortality of a UK isolate of metacercariae kept dry with water added every other day between 10 °C and 25 °C with 95% confidence intervals

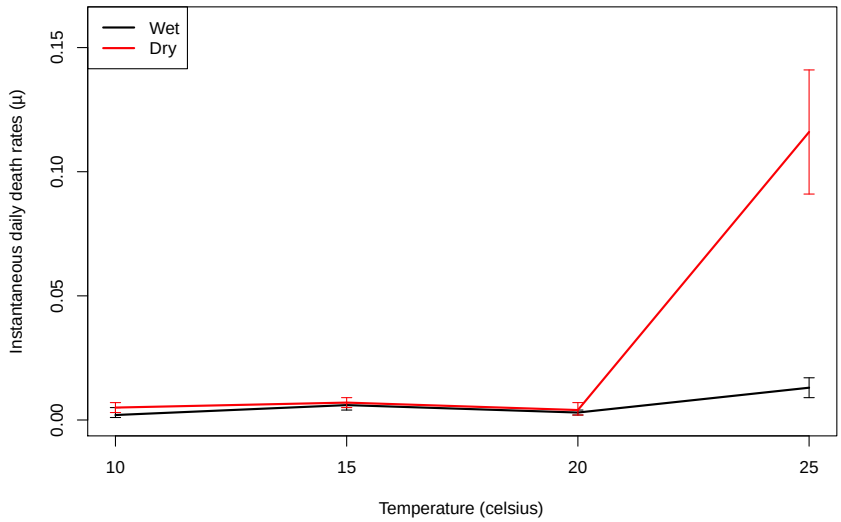


Figure 2.30: Estimated daily death rates of *F. hepatica* metacercariae from the UK at various temperatures under different conditions of water availability with 95% confidence intervals

Table 2.13: Estimated daily death rates of *F. hepatica* metacercariae at various temperatures under different conditions of water availability with 95% confidence intervals

Temperature	In water (95% CI) Mortality	Water added every 2 days (95% CI) Mortality
10 °C	0.002 (0.001-0.005)	0.005 (0.003-0.007)
15 °C	0.006 (0.004-0.007)	0.007 (0.005-0.009)
20 °C	0.003 (0.002-0.004)	0.004 (0.002-0.007)
25 °C	0.013 (0.009-0.017)	0.116 (0.091-0.141)

2.4 Discussion

F. hepatica Eggs

The findings of these experiments with regard to constant temperature egg development are consistent with the published data. This suggests that the parasites' response to temperature in the United Kingdom has not changed significantly over the past 35 years [Boray, 1969, Ollerenshaw, 1971, Al-Habbib, 1974, Al-Habbib and Grainger, 1983]. The consistency with the previous data also implies that it would be reasonable to use both the published data and the experimental data in combination to parameterise the model created in this work. The *F. hepatica* eggs held at varying temperatures between 16 °C and 28 °C had a similar development time (17.90 days) to those at 22 °C, the mean temperature (18.83 days).

Unembronated *F. hepatica* eggs can survive 0 °C for up to 2 weeks without significantly increased mortality (Figure 2.10). Krull [1934] reported that *F. hepatica* could survive for long periods (2.5 years) at 4 °C, which is consistent with the the findings of this thesis at least in the short term, with no increased mortality after 14 days. There is a significant increase in the mortality of eggs kept at temperatures between -2 °C and -8 °C. -7 °C appears to be the lowest temperature that the *F. hepatica* eggs can survive exposure to for two days. Previous reports e.g. Andrews [1999], quoting work done by Vasileva [1960], state that eggs kept at -5 °C are killed within 17 days. Boray [1969] found that after exposure to -15 °C for 24 hours no egg development.

Unembryonated eggs exposed to winter conditions for up to 58 days outside in soil showed no significant increase in mortality. The eggs kept in water had increased mortality over time. The reasons for this are unknown although there may have been contamination

of the water by algae [Boray, 1969]. The eggs in soil were not exposed to free water so would have been less damaged by the effect of freezing temperatures. *F. hepatica* eggs shed onto pasture late in the season, when temperatures have dropped below the lower development threshold of 10 °C, have the potential to survive overwinter and infect snails early in the following year. The anomalously low mortality of observed on day 22 is likely due to a change in the person collecting the data .

F. hepatica Metacercariae

Metacercariae kept in water and metacercariae with water, added every other day, showed similar mortalities at 10 °C, 15 °C and 20 °C of between 0.2% per day and 0.7% per day (Table 2.30). At 25 °C, there was a significant increase in the mortality of metacercariae under dry conditions, increasing to 11.6%. There was also a slight increase in the metacercarial mortality in water (1.3%). The metacercariae from Italy had higher mortality than those from Wales in both the temperature and moisture experiments. This could represent a true difference in the temperature and moisture tolerance of eggs from different parts of Europe. Although this could have also been caused by other factors such as the snail species used to produce the metacercariae, those used at Ridgeway Research may not have been well adapted to the Italian species. In order to fully understand if there is a true difference, a larger number of isolates from different countries would need to be studied.

The effect of longer periods without water on the survival of metacercariae encysted on parafilm and exposed to 20 °C appear to be minimal. There was a marked increase in mortality between metacercariae kept in water and those exposed to dry conditions, although there is little difference in the mortality of metacercariae with water added

every two days and those exposed to 43 days without water. Metacercariae with water added every two days showed similar mortality to the controls in water when exposed to temperatures between 10 °C and 20 °C but showed a far greater mortality at 25 °C.

One of the objectives of the experimental work conducted in this chapter was to provide empirical data to inform the construction of a predictive model for *F. hepatica*. The effects of limited water availability on the mortality of the parasite on pasture appears to be more pronounced at higher temperatures. This will need to be taken into account as climate change predictions are for higher global temperatures, where the mortality of metacercariae may be more strongly affected by water availability.

Chapter 3

A climate-driven mathematical model of *F. hepatica*

3.1 Introduction

Nematode and trematode population dynamics have been modelled since the mid-1900s using different approaches including, geographic information system (GIS), mechanistic and statistical models [Ollerenshaw and Rowlands, 1959, Smith, 2011]. Process-based mechanistic mathematical models, capturing the biology of *F. hepatica*, have not been published. As identified in previous chapters, there is a need for such a model to capture and predict the seasonality of risk periods for animals. Therefore, this chapter will focus on the development of a mathematical model for the free-living stages of *F. hepatica*, using differential equations to describe the transitions between the different stages. It will focus solely on seasonal abundance patterns in *Fasciola* and snail ecology and include the host as a black box. In order to, ultimately, generate the most realistic seasonal model predictions, the host (both domesticated and wild species) would also have to be included. However, modelling the free-living stages of a parasite with such a complex life cycle is a major task in itself. Establishment of the parasite in the host, and host responses, are currently being modelled by others such as [Turner et al., 2016].

Building the model involves translating a set of observed physical phenomena into equations that describe their relationship with, in this case, temperature and rainfall patterns. Differential equation models are a simplified mathematical representation of the true physical life cycle. The stages of the life cycle are described by differential equations that represent the rates at which the parasite or host moves from one stage to the next. In addition, these stages may be affected by environmental factors and these effects also need to be modelled.

The most basic example of a differential equation model is shown in Equation 3.1. It describes how the size of a population (P) changes with time (t). The parameter (k) determines the rate at which the population size changes. If k is greater than one, then the population will grow and if it is less than one the population will decline.

$$\frac{dP}{dt} = kP \tag{3.1}$$

Before building a model, it is important to decide on the level of complexity to be incorporated. A simple model, containing only a few key drivers of parasite epidemiology, might omit some of the biological processes involved yet still be sufficient to answer specific research questions. An advantage of simpler models is that the influence of the individual parameters on model output and performance can easily be analysed; the more parameters a model contains, the more difficult it will be to identify the key drivers. However, in the case of *F. hepatica*, building such a model had not been attempted before and, therefore, it is not known which parameters or equations can be left out. Therefore, there are two basic strategies to choose between when constructing such a model. One approach is to try to include all possible relevant variables in the initial model and then to selectively remove

them if they do not contribute to the predictive capacity in the situation in which the model is being used. An alternative approach is to start with a simple model, involving few variables, and then to add variables sequentially, assessing at each stage if the addition of the variable significantly improves the correlation of the model to validation data. For *F. hepatica*, which has a very complex life cycle and for which the key drivers of the population dynamics are unknown, it was decided to attempt to describe all of the processes and then remove the non-essential elements.

It is important to remember at all times that a model is an approximation of reality. Therefore, it can never claim to incorporate everything that might be important for predicting, for example, parasite abundance. The availability of data on the key parameters that affect each stage in the life cycle (e.g. temperature and rainfall for *F. hepatica*) is of critical importance, as it is only useful to build into a model parameters for which reliable data are available. Also, many parameters can only be researched in the laboratory whereas, at pasture, there will be losses to the population that cannot be estimated. For example, the likelihood of a miracidium finding a snail may be important for parasite success but cannot be parameterised at pasture. Therefore, a model may capture the seasonality of the parasite, and perhaps good and bad fluke years, but never exactly the metacercarial load on pasture at time t. To achieve this, the model would need to be very complex and would require data on factors that are rarely available, such as the elements of micro-environment. Even with simpler models, there will be a difference between the environmental data available and the relevant data for where the parasite resides, which may be important for transmission. For example, there can be a difference of several degrees between the recorded atmospheric temperature and the actual temperature between the grass blades where the parasites and snails reside. With these considerations in mind,

the aim of the model created here is to predict the times of year at which pastures are likely to have a greater number of metacercariae present, in order to inform control strategies. An advantage of the modelling approach taken here is that it takes into account specific interactions between different life stages of the parasite and intermediate host as well as incorporating climatic conditions.

Models may use various time steps. Ollerenshaw and Rowlands [1959] and McIlroy et al. [1990] used month-to-month variation in their model development. To maximise the sensitivity of the model to climatic variables, daily time steps are used here. The Ollerenshaw and Rowlands [1959] and McIlroy et al. [1990] models use climate data from May to October. These months were chosen because of the known epidemiology of the parasite observed in Anglesey and Northern Ireland, respectively. These statistical models are able to predict whether there is relatively more or less transmission in a year compared to other years. However, it has been predicted that climate change might extent the period when transmission takes place [McMichael et al., 2006]. Therefore, it is important to have a model that can adapt to these predicted changes.

The data that are used to develop a model may be gathered from laboratory and field experiments which are used to investigate the factors that affect different stages of the parasite life cycle. Different models can then be validated against real world data such a pathology observed in abattoirs to see to what extent the predictions made by the models correlate with what is seen in reality. However, there are challenges to the validation process. Often, there are few relevant data collected in a real world setting with which to validate the model [Smith, 2011]. In addition, in the field, it is often the case that what is measured is what is practical to measure, which may not necessarily be what is needed to validate the model. For example, the model might predict snail abundance, which will

include snails that are in the soil but not readily visible, but data on snail collections will generally focus on visible snails. Also, snail populations are normally geographically dispersed in specific habitats, which makes it hard to know which part of a field to sample. Sampling the same area of pasture longitudinally over time may partially get around this, but if a low density sample site was chosen, then measured population sizes may still be unreliable [Smith, 2011].

From the outset, it is therefore clear that both the modelling of such a complex life cycle and the validation of model output will pose significant challenges. However, even if the model cannot capture reality, in terms of exact parasite and snail abundance very accurately, shifts in epidemiology predicted by a model, for example in relation to future climate change, may be informative. Furthermore, it would appear that process-based models are the only realistic way forward in terms of both testing the effect of control programmes and designing new control strategies for the future.

The differential equation model is capable of incorporating changing climate conditions over short time periods. Climate change is predicted to cause more extreme weather events [Watson et al., 1997] which could be incorporated into the model in a more satisfactory manner. The model that has been developed here can potentially be used as a tool in the design of control programmes for *F. hepatica*. For example, individual parameters can be manipulated to allow for changes in farm management practices, such as the timing of anthelmintic treatment. Most of the parameterisation of the model was conducted using data gathered on fluke from the UK and Northern Europe. There is potential for the model framework to be applied to different areas of the world if reliable data are available on the development and mortality of the parasite and its hosts in those regions.

3.2 Material and Methods

3.2.1 Model structure

A differential equation model was developed to predict risk periods for animals, related to *F. hepatica* metacercariae on pasture based on temperature and rainfall data. The model incorporates the life cycles of *F. hepatica* and *G. truncatula* and uses the effects of environmental conditions on the free-living stages of the parasite and its snail host to predict the quantity of metacercariae on pasture throughout the year. The parasite life stages that are explicitly modelled are the egg and metacercariae. The miracidia and the in-snail development of the parasite (sporocyst, radiae and cercariae) are not explicitly modelled individually. In other words, by analogy with the ruminant host, the snail intermediate host is also modelled as a black box, with snail abundance but not snail immunity/resistance part of the model. The reason for this is because the miracidia and the cercariae have very short lifespans in the environment (less than one day) [Al-Habbib, 1974]. In-snail development is modelled as the infected snail stage, with outputs soley temperature and rainfall dependent. The snail stages explicitly modelled are the egg, juvenile snail, mature snail and infected snails. These categories were chosen because snails do not start producing eggs until they reach maturity (around 4mm in size) and there is a castration effect when snails are infected [Wilson and Denison, 1980]. In this model, only the mature snails produce eggs, all other stages do not [Heppleston, 1972].

The model was parametrised using published data from peer reviewed journals, PhD and Masters thesis and laboratory work by the author and work conducted by Ridgeway Research to provide data for this PhD (see section 3.2.3 for details). The variables and parameters used in the model are given in Table 3.1. The model framework is presented

Table 3.1: Variables and parameter model definitions

Variables/ Parameters	Definition	Value
E	Fluke eggs on pasture	-
M	Metacercariae on pasture	-
S_e	Snail eggs	-
S_j	Juvenile snails	-
S_m	Mature snails	-
S_i	Infected snails	-
δ_e	Development rate from egg to miracidium on pasture	Instantaneous daily rate
δ_{Se}	Development rate of snail eggs	Instantaneous daily rate
δ_{Sj}	Development rate of juvenile snails	Instantaneous daily rate
μ_e	Mortality rate of fluke eggs on pasture	Instantaneous daily rate
μ_m	Mortality rate of metacercariae on pasture	Instantaneous daily rate
μ_{Se}	Mortality rate of snail eggs	Instantaneous daily rate
μ_{Sj}	Mortality rate of uninfected juvenile snails	Instantaneous daily rate
μ_{Sm}	Mortality rate of uninfected mature snails	Instantaneous daily rate
μ_{Si}	Mortality rate of infected snails	Instantaneous daily rate
λ_{Sm}	Per capita daily fecundity of mature snails	Eggs / snail / day
c	Rate of shedding of metacercariae by infected snails	Metacercariae / infected snail / day
A	Proportion of pasture that is suitable snail habitat	Proportion
R	Proportion of available habitat used per snail per day (estimated from mean snail ranging behaviour + available habitat size)	Proportion
i	Rate of ingestion of metacercariae on pasture	Instantaneous daily rate
ϵ	Establishment rate of miracidia in snails (proportion of contacts yielding cercariae)	Proportion

in Figure 3.1 and described in Equations 3.2(a-f). *F. hepatica* eggs are represented in the model by E. At each time step, a proportion of the parasite eggs develop (δ_e) and become infectious to juvenile and mature snails and a proportion die (μ_e). As this is not a full cycle model, parasite eggs are added into the model at every time step (E_{new}) to simulate shedding from infected mammalian hosts (Equation 3.2a).

Snail eggs (Se) can develop (at a rate δ_{Se}) into juvenile snails (Sj) or they can die (at a rate μ_{Se}). Mature snails (Sm) produce eggs (Se) at a rate which is determined by the fecundity of the mature snails (λ_{Sm})(Equation 3.2b). Juvenile snails (Sj) develop into mature snails (Sm) at a rate of δ_{Sj} or die, at a rate of μ_{Sj}. They become infected from hatched parasite eggs (δ_e) at the rate of δ_eE×A×R×$\epsilon \times S_j$ (see below). *Fasciola* egg hatching is not explicitly modelled, once development is completed, the eggs hatch. Juveniles that are infected do not develop into the mature snail category (S_m) but into infected snails (S_i) (Equation 3.2c). Uninfected juvenile snails develop into mature snails, which do not undergo further development. Mature snails can also become infected by the parasites at a rate of δ_eE×A×R×$\epsilon \times S_m$ or they die at the rate of μ_{Sm} (Equation 3.2d).

For a snail to become infected by a hatched parasite egg (δ_e), both the host and the parasite need to be found in the same areas (A) and the parasite needs to encounter a susceptible snail (R) (Equation 3.2c-d). This infection is influenced by the probability of the parasite establishing within the snail (ϵ). Snails enter the infected category (S_i) from both the juvenile and mature snail groups at the rate of δ_EE×A×R×$\epsilon \times (S_j + S_m)$ and die at a rate of μ_{Si}. The infected snails shed metacercariae (M) determined by their shedding rate (c) 3.2e). The metacercariae produced by the infected snails do not undergo any further development, they die at a rate of μ_M or they are ingested by a mammalian host (i)).

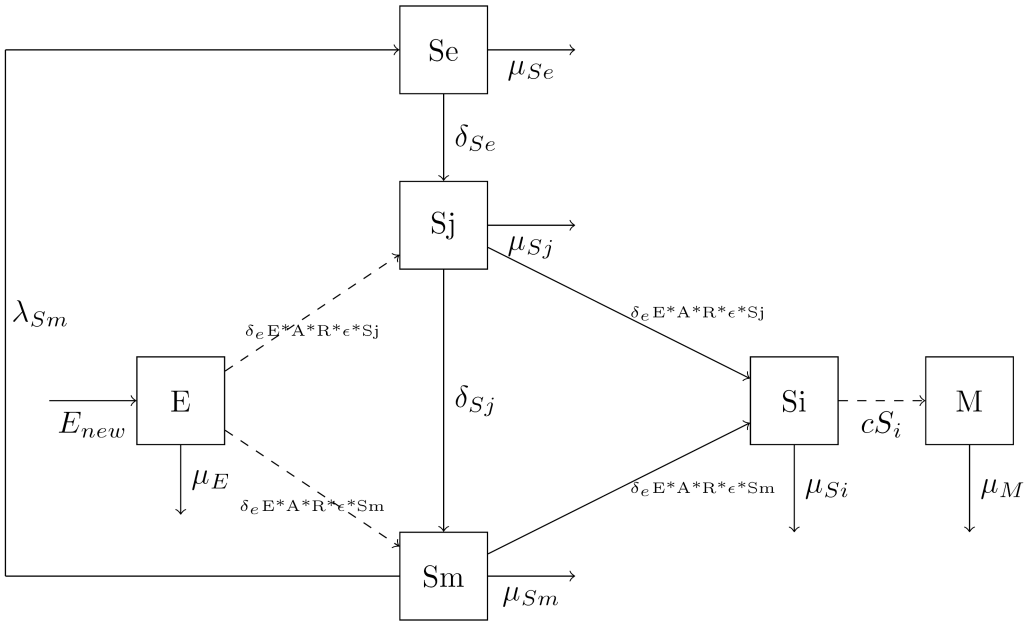


Figure 3.1: Model diagram. Solid lines represent development of organisms (arrows connecting boxes) and mortality (arrows unconnected to boxes), dashed lines represent parasite transmission.

$$\frac{dE}{dt} = -(\delta_e + \mu_e)E + E_{new} \tag{3.2a}$$

$$\frac{dS_e}{dt} = -(\delta_{Se} + \mu_{Se})S_e + \lambda_{Sm} \tag{3.2b}$$

$$\frac{dS_j}{dt} = -(\delta_{Sj} + \mu_{Sj})S_j + \delta_{Se}Se - (\delta_e E \times A \times R \times \epsilon \times S_j) \tag{3.2c}$$

$$\frac{dS_m}{dt} = -\mu_{Sm}S_m + \delta_{Sj}S_j - (\delta_e E \times A \times R \times \epsilon \times S_m) \tag{3.2d}$$

$$\frac{dS_i}{dt} = -\mu_{Si}S_i + (\delta_e E \times A \times R \times \epsilon \times (S_j + S_m)) \tag{3.2e}$$

$$\frac{dM}{dt} = -(\mu_m + i)M + cSi \tag{3.2f}$$

Differential equation model for the external life cycle of *F. hepatica*. 3.2a fluke egg, 3.2b snail egg, 3.2c juvenile snails, 3.2d mature snails, 3.2e infected snails, 3.2f fluke metacercariae.

3.2.2 Model assumptions

There are several assumptions underpinning the model. The assumptions relate to the biology and ecology of the parasite and the snail host. Where possible, the validity of the assumptions were corroborated using published data on the subject. Where published data were not available, experts were consulted who had either worked with and studied *F. hepatica* or who were veterinarians with specific knowledge about the disease.

One important assumption is that fluke eggs are deposited evenly on pasture and, therefore, many eggs are deposited in locations without suitable snail hosts. Experiments determining the spread of bovine faecal matter naturally deposited on pasture might provide a proxy for egg deposition. Researchers found that each dung pat could spread over an area of over 13m^2 [Boswell and Smith, 1976]. Boswell and Smith [1976] hypothesised that a similar pattern would be observed for ovine faeces. It has been observed that a cow defaecates an average of 8.1 times a day, while a calf defecates an average of 6.2 times a day [Stromberg, 1997] and sheep defecate more frequently between 19 and 26 times a day [Williams and Haynes, 1995]. Beetles and earthworms contribute significantly to the breakup and distribution of faeces around the pasture. Cattle trampling faeces will also serve to disperse faecal material [Stromberg, 1997]. The degradation and dispersal of faeces through insect activity and rainfall allows the eggs to escape the faeces and to develop as fluke eggs do not develop until clear of faeces. Cattle do not graze in a random pattern but often in set patterns [Bryan and Kerr, 1988]. Sheep pellets degrade more rapidly taking three to six weeks compared to cattle faeces which took upto 12 months [Williams and Haynes, 1995]. The model does not contain a spatial component to model the parts of pasture suitable for snails, it does not predict where the snails are. The distribution

of snails on pasture is unknown, and adding within-pasture grazing behaviour would add too much complexity to the model. However, through an area function, it is possible to model the part of pasture suitable for snail habitation as a proportion, which has been made a function of rainfall. Increased rainfall, therefore, increases the area in which snails can be found and, therefore, the proportion of hatched eggs being able to find and infect a suitable snail [Vignoles et al., 2011].

The intermediate snail hosts are assumed to be confined to a limited area of suitable habitat within the pasture. The preferred habitat of *G. truncatula* is the muddy regions around temporary water bodies, areas with poor drainage and streams. The area of suitable habitat for the snails would be expected to change with rainfall, increased rainfall being assumed to enlarge the areas suitable for snails. In the model, only mature uninfected snails (larger than 4mm high) are assumed to produce snail eggs [Nice, 1979]. Once a snail becomes infected with *F. hepatica*, there is a castration effect and they stop producing snail eggs [Wilson and Denison, 1980].

There are no published papers on the natural contact rates between miracidia and snails in the environment. From laboratory experiments on miracidia, it is known that they die within 8 to 24 hours of emergence from eggs [Boray, 1969] . Therefore, their lifespan is not explicitly modelled and is assumed to be one day, and the contact area between snails and eggs is modelled to approximate the probability of infection. Thus, the availability of miracidia is represented by the number of eggs which have developed in each daily time step. The daily contact rate between snails and miracidia is a product of the number of miracidia on pasture, the proportion of pasture that is suitable for snail habitat and the proportion of those habitats that snails will use each day, thus giving the number of miracidia that each snail is likely to come into contact with each day.

The proportion of snails infected is dependent, therefore, on the density of miracidia and snails on pasture. Cercariae are assumed to be shed from infected snails at a constant daily rate, although in reality this shedding occurs approximately every 7 days [Vignoles et al., 2006]. When considering large numbers of snails, this periodicity of shedding will not be a significant factor in timing of cercarial shed and, therefore, has been omitted from the model.

The model assumes that diurnal temperature fluctuations do not significantly accelerate development rates [Al-Habbib, 1974]. Experiments conducted by Al-Habbib and Grainger [1983] demonstrated that varying daily temperatures between 16 °C and 28 °C did not significantly accelerated the development of the eggs. This was confirmed in similar experiments carried out and described in Chapter 2. According to the literature, temperatures less that 9.5 °C and greater than 30 °C stop development for all the free-living stages of the parasite. Temperatures above 30 °C result in high levels of mortality [Al-Habbib, 1974]. In the model, apart from affecting the snail habitat area, rainfall is considered to affect the mortality of parasite and snail stages. Overall, temperature affects the development rate, therefore, temperature is the primary driver of seasonality, whereas rainfall, alongside temperature, is a key determinant of abundance levels, and hence the intensity of infection seen in sheep and cattle.

3.2.3 Model parameterisation

It was intended for the model to be applicable over the whole of Europe. This limited the available input variables that could be used in the model. The most widely accessible data were for temperature and rainfall. For rainfall, there were two options, either to use the number of rain days or the volume of rain. The decision to use the number of rain days was made because of several previous studies that showed that this had a large impact on the levels of infection observed [McCann et al., 2010]. The relationship between rainfall volume and water availability on the ground is influenced by soil-type, drainage and the slope of the ground but could not be included within the parametrisation.

A literature search was conducted looking for published papers on the effect of temperature and moisture on the parasite and its intermediate host and on development and survival under laboratory or field conditions. Seven papers were identified reporting laboratory controlled studies into the effect of temperature on development and survival of *F. hepatica* and *G. truncatula*. The data from these papers were extracted from tables where available, and where the data were only presented in figures, WebPlotDigitizer [Rohatgi, 2015] (accessed 10/2/2015) was used to acquire the required data points. For the development of the parasite and snail host, the time in days for 50% to develop was measured. The inverse of the time taken was then used to calculate the daily development rates. The earliest paper found was Roberts [1950], which focused on *G. truncatula* development, and the most recent work was a PhD thesis, Nice [1979], which examined both the parasite and the host. No more recent laboratory studies on the effect of temperature and water availability on the parasite and host were found. All of the studies identified were conducted using isolates from the United Kingdom or Ireland.

The data obtained from these publications were imported into R version 3.0.2 [R Core Team, 2013] for the analysis. Three models were considered to describe the relationships between temperature and development or mortality for each parameter; a linear model, a 2nd order polynomial and a 3rd order polynomial. These were selected as previous studies had used similar models and it seemed likely that they would best fit the data. The best approximation model was selected using Akaike information criterion (AIC).

$$-2L_m + 2m$$

Where L_m is the maximum *log-likelihood* and m is the number of parameters in the model. One of the advantages of AIC over other statistical tests is that it takes into account both the statistical goodness of fit and the number of parameters.

In the model, the area in which snails are found is influenced by the number of rain days (days with more than 2mm of rain) over the preceding 14 days. The length of time which rainfall affected was achieved by using the VIDA database to validate the effect of rainfall on the model prediction. Under wet conditions, there is a higher rate of contact between snails and intermediate host, which results in higher rates of infection and greater numbers of infected snails. The parametrisation of rainfall of the effect of rain fall was challenging as the available data on the relationship between specific parasite and snail stages and rain fall is minimal.

F. hepatica egg development (δ_e)

Data on the development of *F. hepatica* eggs were obtained from several papers [Rowcliffe and Ollerenshaw, 1960, Al-Habbib, 1974, Connolly, 1979] and the experimental work presented in Chapter 2 (Figure 3.2). These studies were conducted under laboratory conditions using temperature-controlled incubators. The eggs were incubated in water to eliminate the effect of increased mortality caused by desiccation. The eggs were examined over the course of a month to determine when 50% of the viable eggs had hatched. The relationship between temperature and *F. hepatica* egg development rate (δ_E) between 10 °C and 30 °C, was found to be well described by a linear function (Figure 3.2); the equation is given in Table 3.2. At temperatures below 10 °C, there is no development of the parasite egg, so the development rate was assigned as zero in the model.

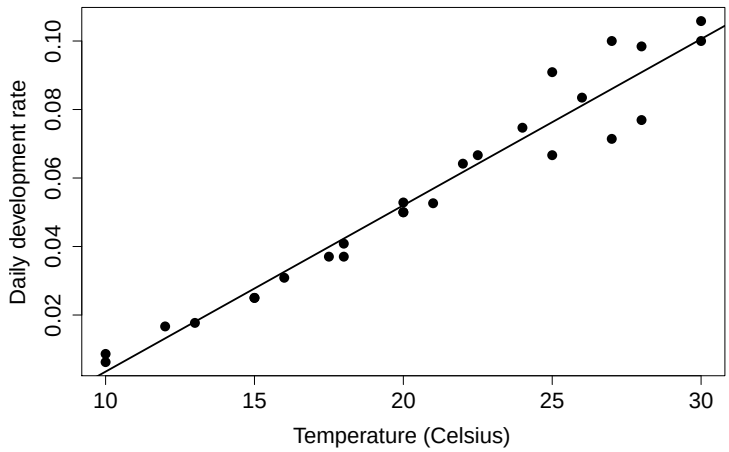


Figure 3.2: The rate of development of *F. hepatica* eggs (δ_e) at different temperatures, combining thesis data together with published data from Roberts [1950], Kendall [1953], Al-Habbib [1974], Nice [1979]

F. hepatica egg mortality (μ_e)

Fewer studies have investigated the effect of temperature on *F. hepatica* egg mortality compared to development. Al-Habbib [1974] reported the effect of temperature on egg mortality (Figure 3.3) between 10 °C and 30 °C. Several studies have reported extremely high mortality in eggs that are kept above 30 °C for even a short time [Al-Habbib, 1974]. In our model, if the temperature rises above 30 °C, then the mortality of the eggs is assumed to be 100%.

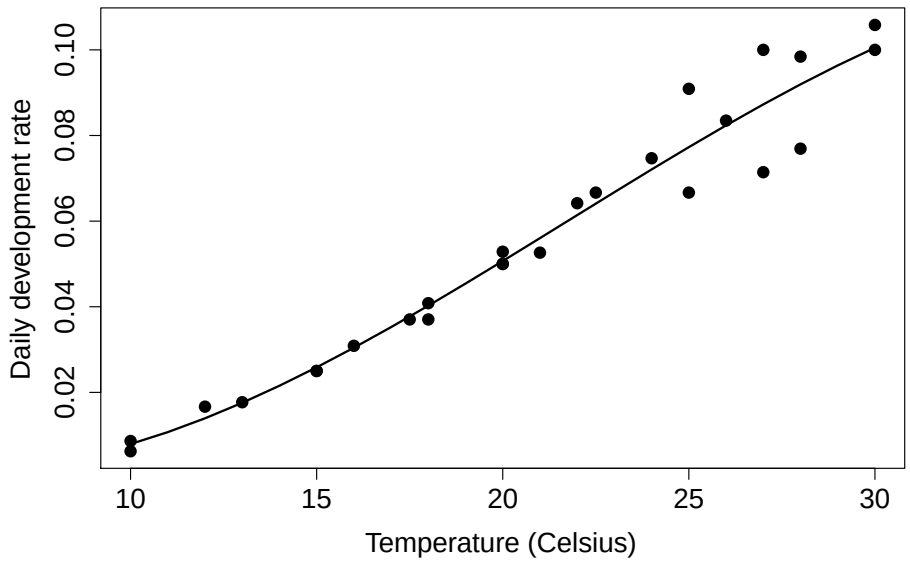


Figure 3.3: *F. hepatica* egg mortality rate (μ_e) at different temperatures , combining thesis data together with published data from Roberts [1950], Kendall [1953], Al-Habbib [1974], Nice [1979]

G. truncatula egg development (δ_{Se})

As *G. truncatula* is the primary intermediate host for *F. hepatica* in Europe, several studies have been conducted on the development of the snail eggs at various constant temperatures (Figure 3.4) [Roberts, 1950, Kendall, 1953, Al-Habbib, 1974, Nice, 1979]. The eggs in these studies were kept in water in the respective laboratories. A linear relationship is seen between temperature and *G. truncatula* egg development (Table 3.2c).

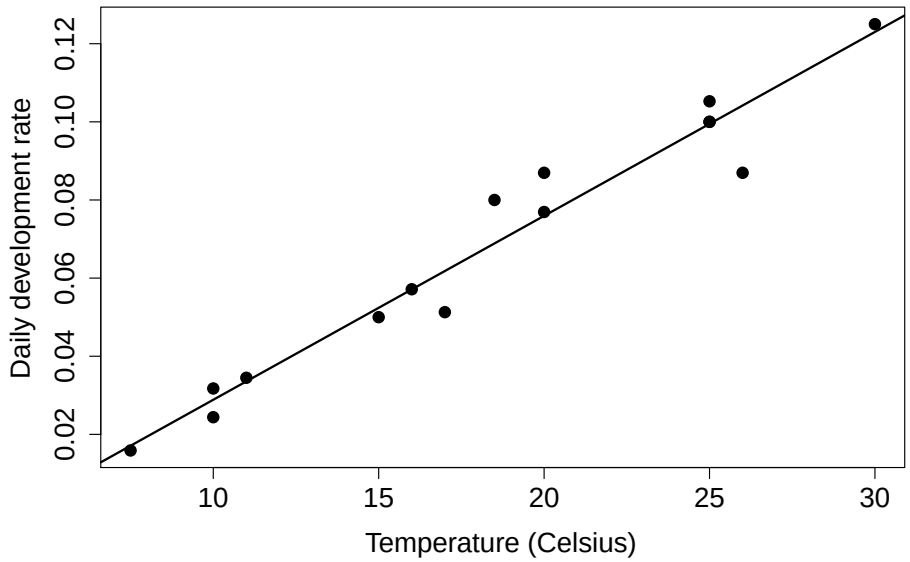


Figure 3.4: The rate of *G. truncatula* egg development (δ_{Se}) at different temperatures (Roberts [1950], Kendall [1953], Al-Habbib [1974], Nice [1979])

G. truncatula egg mortality (μ_{Se})

Nice [1979] examined over 6500 *G. truncatula* eggs to determine the mortality at 10, 15, 20 and 25 °C (Figure 3.5). These were the only data found in the literature on the mortality of snail eggs. In themselves, 4 data points would not appear to be enough to give a good description of the relationship. However, given the number of eggs examined by Nice [1979], the confidence in the individual data points is high. There is uncertainty as to whether the true relationship between temperature and mortality is linear (Table 3.2d). Above 30 °C, there is very high egg mortality [Roberts, 1950], in our model it is assumed to be 100%.

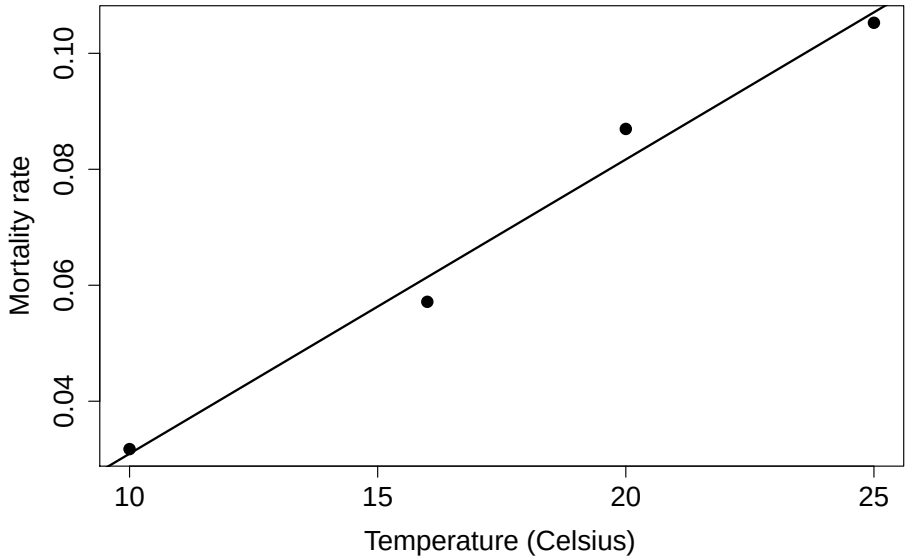


Figure 3.5: The rate of *G. truncatula* egg mortality (μ_{Se}) at different temperatures (data from [Nice, 1979])

G. truncatula development to adult snails (δ_{Sj})

There are limited published data on the development of *G. truncatula* kept at constant controlled temperatures between 10 °C and 30 °C. In most studies, the snails are maintained at room temperature of (around 20 °C) as this is seen as the optimal temperature for a stable colony [Nice, 1979]. The most comprehensive study was conducted by Nice [1979], who looked at snail growth at temperatures of 5, 10, 16, 20 and 25 °C. It was found that there is negligible snail development at 5 °C and there is a linear relationship between growth and temperature from 10 °C to 25 °C (Figure 3.6 and Table 3.2e).

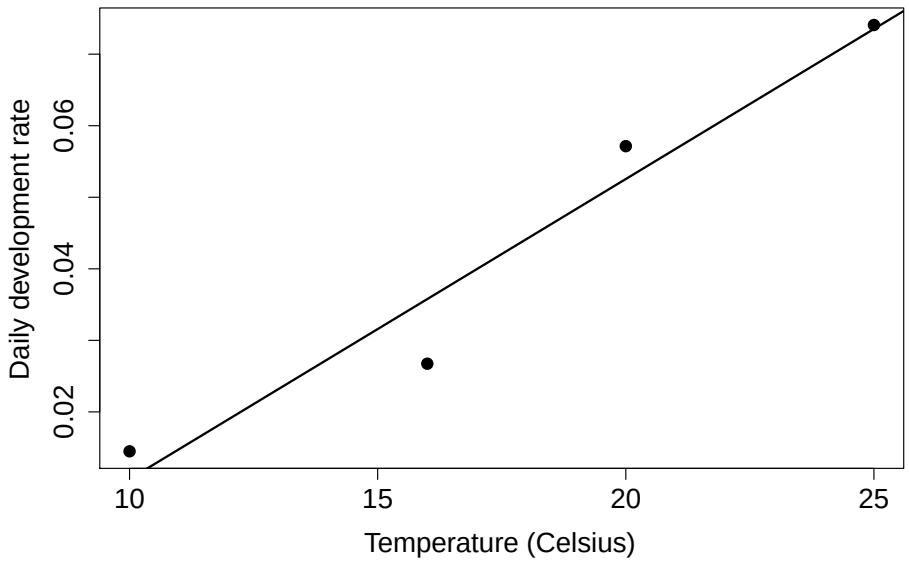


Figure 3.6: The rate of *G. truncatula* development (δ_{Sj}) at different temperatures (data from [Nice, 1979])

G. truncatula adult mortality (μ_{Sj}, μ_{Sm})

Similar to the development of *G. truncatula*, there are relatively little data available on the mortality of snails at different temperatures. Nice [1979] studied snail mortality at 5 constant temperatures between 5 °C and 25 °C and found that the mortality of the snails rose as the temperature increases. The change in mortality with temperature is linear (Figure 3.7).

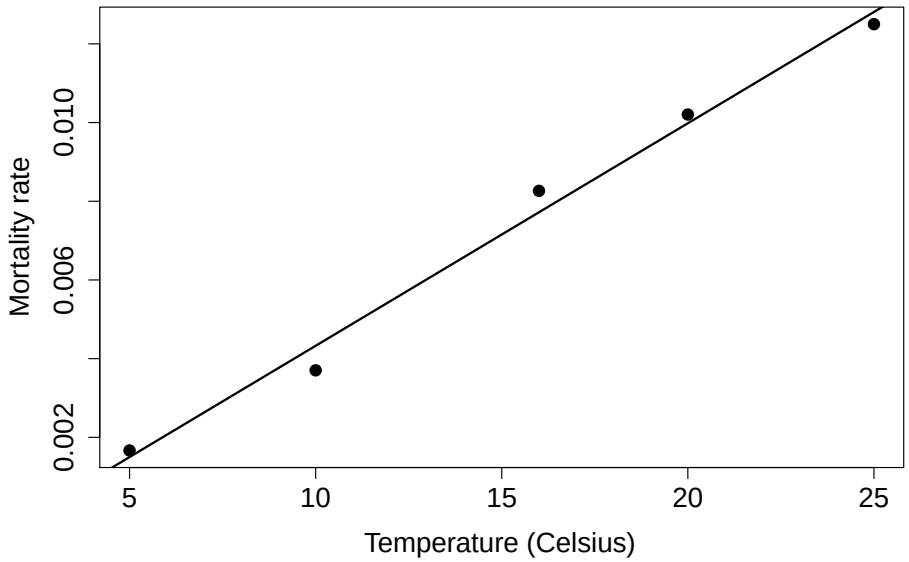


Figure 3.7: The rate of *G. truncatula* mortality (μ_{Sj},μ_{Sm}) at different temperatures (data from [Nice, 1979])

F. hepatica metacercarial mortality (μ_m)

To parametrise the mortality of *F. hepatica* metacercariae, data gathered by Boray and Enigk [1964] were combined with additional data gathered as part of this thesis (see Chapter 2). The relationship was represented by the quadratic line presented in Figure 3.8, (Table 3.2g), although there is considerable variation in mortality at higher temperatures. The mortality of the metacercariae is also influence by water availability, using the data gathered in Chapter 2.

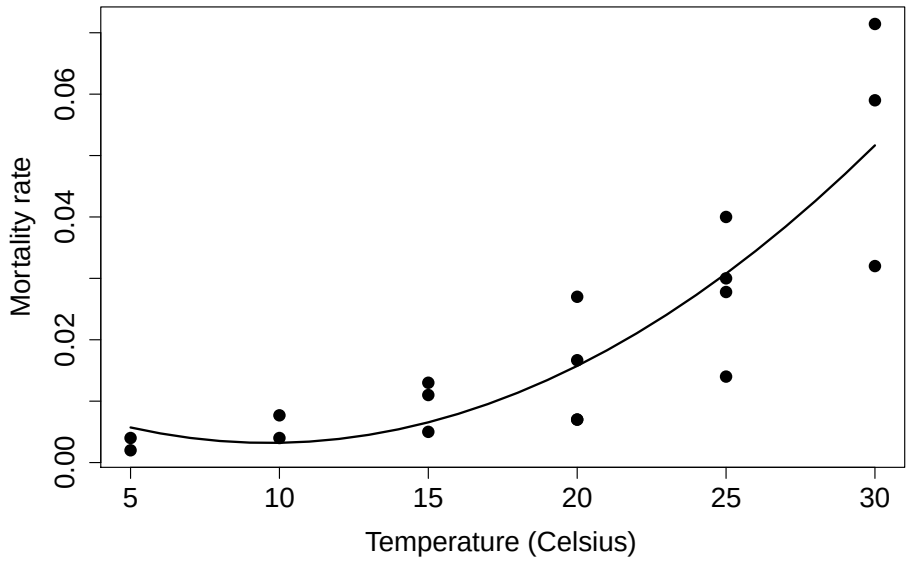


Figure 3.8: The rate of metacercariae mortality (μ_M) at different temperatures (data from [Boray and Enigk, 1964]) and experimental work

Table 3.2: The equations generated from published data and experimental work as part of this thesis for the parameterisation of the model

Parameter	Equation	Adjusted R-squared	p-value	No.	Figure
F. hepatica egg development[a]	$\beta_E = 0.00485548 \times T - 0.0450860$	0.9473	<0.001	26	Figure 3.2
F. hepatica egg mortality [b]	$\mu_E = 0.014 - 6e\text{-}4T + 3.28e\text{-}5T^2 - 4.74e\text{-}7T^3$	0.9351	<0.001	10	Figure 3.3
G. truncatula egg development [c]	$\beta_{Se} = 0.0047080 \times T - 0.018225$	0.9491	<0.001	15	Figure 3.4
G. truncatula egg mortality [d]	$\mu_{Se} = 0.0047080 \times T - 0.0182255$	0.9764	0.008	4	Figure 3.5
G. truncatula development [e]	$\beta_S = 0.0041946 \times T - 0.0313423$	0.921	0.027	4	Figure 3.6
G. truncatula mortality [f]	$\mu_{Se} = 0.0005655 \times T - 0.001328$	0.9859	<0.001	5	Figure 3.7
Metacercarial mortality [g]	$\mu_M = 0.0140 - 0.0023T + 0.00012T^2$	0.7283	<0.001	18	Figure 3.8

[a] Rowcliffe and Ollerenshaw [1960], Al-Habbib [1974], Connolly [1979] and data gathered as part of this thesis
[b] Al-Habbib [1974]
[c] Roberts [1950], Kendall [1953], Al-Habbib [1974], Nice [1979]
[d] Nice [1979]
[e] Nice [1979]
[f] Nice [1979]
[g] Boray and Enigk [1964] and data gathered as part of this thesis

3.2.4 Model validation

Validating the model against real world observations is an important step in assessing the usefulness of my model. The different stages of the model were validated using data obtained from published studies with a longitudinal series of data. There are no published studies on the number of either *F. hepatica* or *G. truncatula* eggs present in the environment, so validation could not be conducted on these stages. Several studies have investigated the abundance of *G. truncatula* in various locations, including Ireland [Relf et al., 2011], Wales [MacKintosh and Brophy, 2012], Belgium [Charlier et al., 2014a], and the Netherlands [Gaasenbeek et al., 1992]. Due to the limited number of studies undertaken to identify the presence of metacercariae in the environment [Gaasenbeek et al., 1992], data on acute animal infections were also used as a proxy for metacercariae in the environment. To compare with the model results, it was necessary that temperature and rainfall data were available for the areas and time when the data were gathered. The observations were compared to the model output to investigate the extent of correlation between the retrospective observational data and the model predictions.

Validation of the snail stages

There are few studies available that measure the temporal variation in snail populations alongside associated collection of environmental data. Some of the studies were quite small, in terms of the numbers of snails collected, and thus the results are associated with considerable statistical uncertainty. Three papers from studies in Europe were used to try to validate the snail portion of the model, namely, Gaasenbeek et al. [1992], Relf et al. [2011] and Charlier et al. [2014a]. In these studies, longitudinal data were collected on the presence of *G. truncatula* in a given geographic area, although the data collection methods

differed. Gaasenbeek et al. [1992] gathered data in the the Netherlands by searching 20 randomly chosen 20 x 50 cm areas of drainage ditches approximately every six weeks. Relf et al. [2011] sampled several known snail habitats on a farm in Ireland using a 10m transect which was searched for 30 minutes between 8 a.m. and 11 a.m on sampling days. by the same person monthly between 2006 and 2008. Charlier et al. [2014a] searched four farms in Belgium for *G truncatula* monthly between April and November 2012, using a 10m transect with a 15 minute search time. The number of snails found at any one time point during the studies varied substantially between the studies. Due to the very low average number of snails found by Gaasenbeek et al. [1992], it was difficult to assess the effect of seasonality on the snail population. The effect of seasonality was clearer in the data gathered by Relf et al. [2011], as the sampling method was such that a larger number of snails was found and the sampling was more systematically applied. Our model predicts the snail population for a whole pasture plot daily, whereas the data used for the validation of the snail part of the model was done by sampling known snail habitats for 10 minute periods on a limited number of days, as it is not possible to assess the total number of snails in a given area due the difficulty in locating the snails. It is, therefore, also not known how much of the differences in snail numbers found can be explained by random, or temperature/rainfall induced, variation.

Rainfall and temperature data were obtained for areas close to the study sites (no more that 25 km away) for the relevant years of the published studies. These were then input into our model to compare the model prediction to the observations from the published work. Gaasenbeek et al. [1992] determined the number of metacercariae per square metre of pasture at the at the same locations as the snail studies.

Model validation using VIDA data

In the United Kingdom, passive surveillance for fasciolosis is carried out by the Animal and Plant Health Agency (APHA), who are sent animals suspected of being infected for diagnosis. Reports are published each year on the number of acute and chronic fasciolosis cases diagnosed. This is referred to as Veterinary Investigation Diagnosis Analysis (VIDA) and is published by county. For validation, cases of acute fasciolosis in sheep diagnosed by the APHA were used because these cases are caused by a rapid intake of *F. hepatica* metacercariae and better reflect the infection risk for grazing livestock. Due to the limited number of cases reported in the VIDA data, rather than using the county data, the total counts for Wales, Scotland and the South West of England was used. Temperature and rainfall data was obtained for central locations in Wales, Scotland and the South West of England. The model was run using these data to produce estimates of the number of metacercariae on pasture throughout the year. As the model output was metacercarial number on pasture, whereas the VIDA data reports acute disease in sheep the metacercarial predictions 2 months before the VIDA data were used, to allow for the progression of disease to be reported in sheep.

3.3 Results

3.3.1 Model output

Figure 3.9 shows some typical model output, run on temperature and rainfall data from Wales, in this case, for the years January 2006 to December 2007. The model has 6 categories that are predicted daily namely, *Fasciola* eggs and metacercariae and the four snail stages, egg, juvenile, adult and infected snails. In Wales, the model predicts that there is a build-up of *Fasciola* eggs on pasture early in the year, until mid March when temperatures begin to rise and the eggs start to develop. This leads to an increase in the number of infected snails throughout the summer. After the infection has matured inside the snails a sharp rise in the number of meatcercariae on pasture is seen between July and October. The number of snail eggs on pasture appears to fluctuate throughout the year, but with increased numbers seen in March as the mature snails become more active and begin egg shedding. The warmer temperatures in March cause the snail eggs to develop into juvenile snails and rapidly mature into adult snails. The snail numbers throughout the year are limited by the carrying capacity of the pasture, which in the model is set to 500 snails, as the density of snails increases, the fecundity of the snails become decreases. There is some between-year variation in the number of metacercariae between 2006 and 2007, 2006 has a lower peak but more metacercariae are seen throughout the year. Similar patterns in the populations are seen between Wales and Scotland (Figure 3.10) although, for these years, far fewer metacercariae are predicted under Scottish climate conditions. Figure 3.11 shows the model output for the South West of England for the same years, fewer metacercariae are predicted to be present compared to Wales but it predicts that there will be a low level of metacercariae on pasture almost year-round.

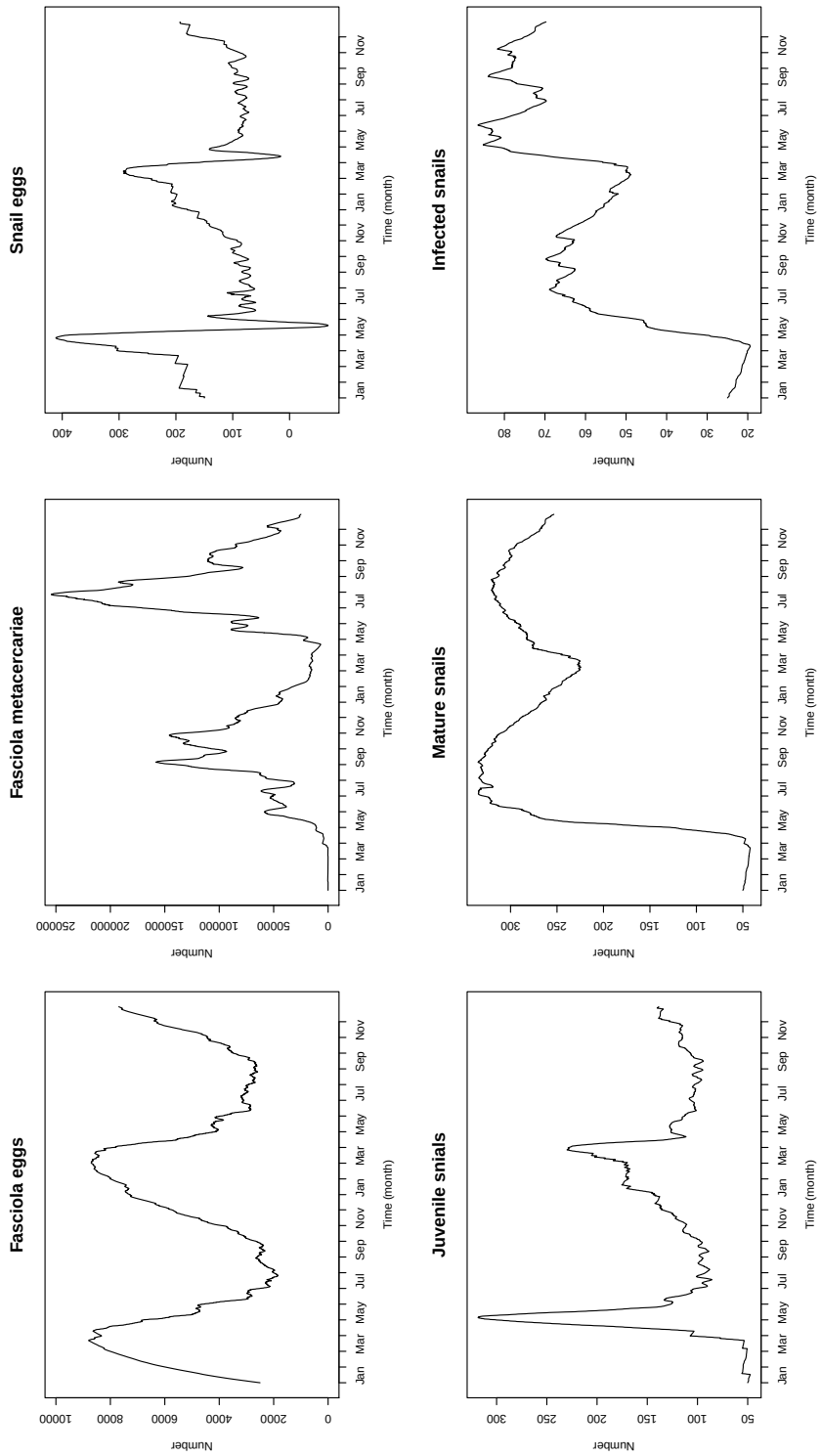


Figure 3.9: Model predictions for all parasite and snail stages between 2006 and 2007 in Wales

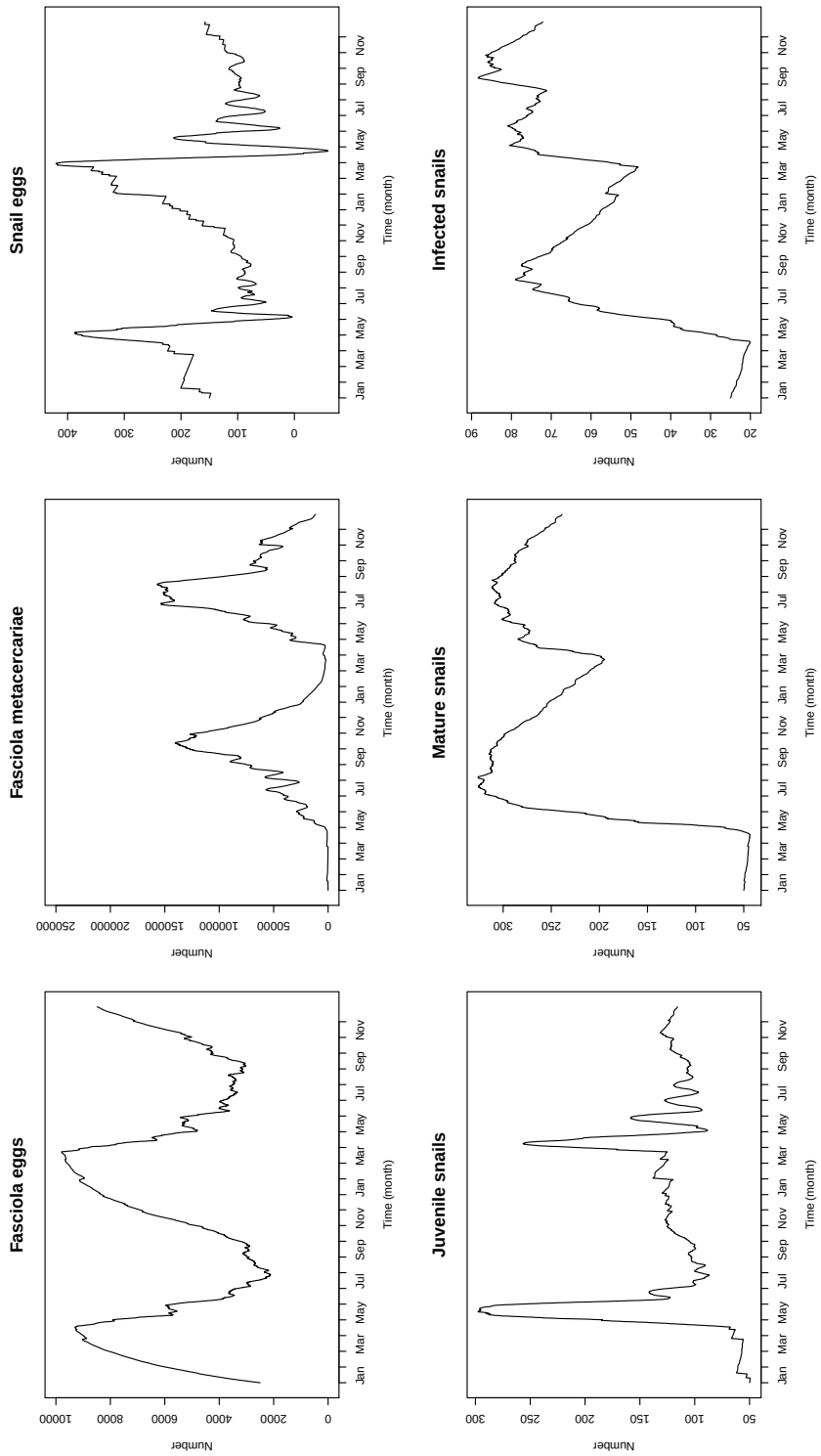


Figure 3.10: Model predictions for all parasite and snail stages between 2006 and 2007 in Scotland

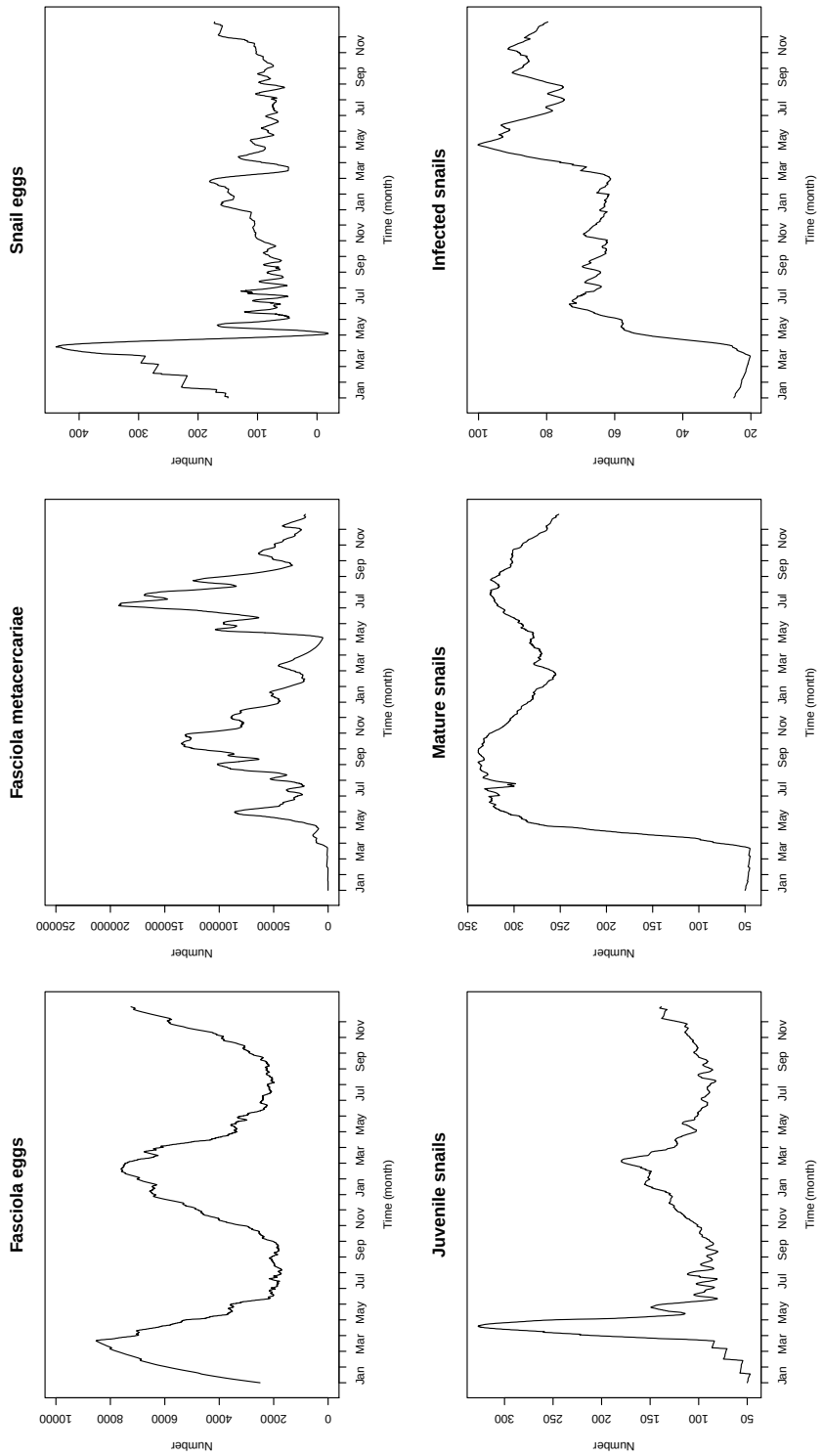


Figure 3.11: Model predictions for all parasite and snail stages between 2006 and 2007 in South West England

3.3.2 Validation

Figures 3.12 and 3.13 show the number of snails predicted by the model compared to the number of snails reported in Gaasenbeek et al. [1992] between 1987 and 1989.

Figure 3.12 shows the number of snails reported in Gaasenbeek et al. [1992] between 1987 and 1989, in red, and the model prediction for the number of snails present. A linear regression was used to examine the correlation between the model and the experimental data. Figure 3.12 shows the daily model prediction of the number of snails present (solid black line) alongside the number of snails reported in [Gaasenbeek et al., 1992] on the dates data were collected. There are between 5 and 6 observations of snail numbers each year between February and December.

The number of snails found over the study period varied between 1 and 15 during any one collection. There was considerable in the number of snails found between temporally close time points. Note that the model predicts the total number of snails in a defined area, whereas the data of Gaasenbeek et al. [1992] relate to the mean number of snails per square meter. Thus, it would not be expected that the counts and predictions are similar but the validation is based on whether the variations in the observed data are mirrored in the model predictions. In this case the correlation between the observed and predicted snail density appears to be poor (Figures 3.12 and 3.13).

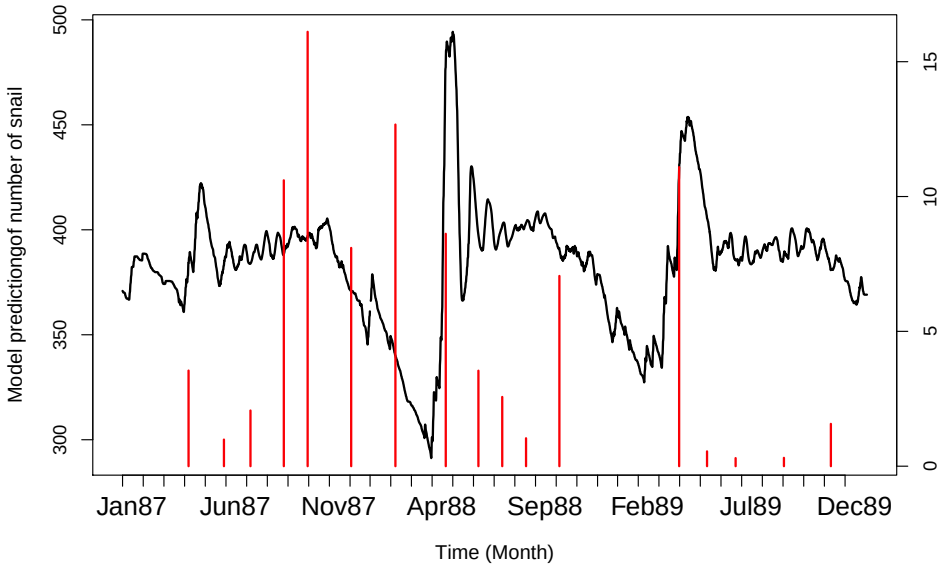


Figure 3.12: The model-predicted snail population (left y-axis, black line) and snail observations made by Gaasenbeek 1992 in the Netherlands (right y-axis, red bars) between 1987 and 1989

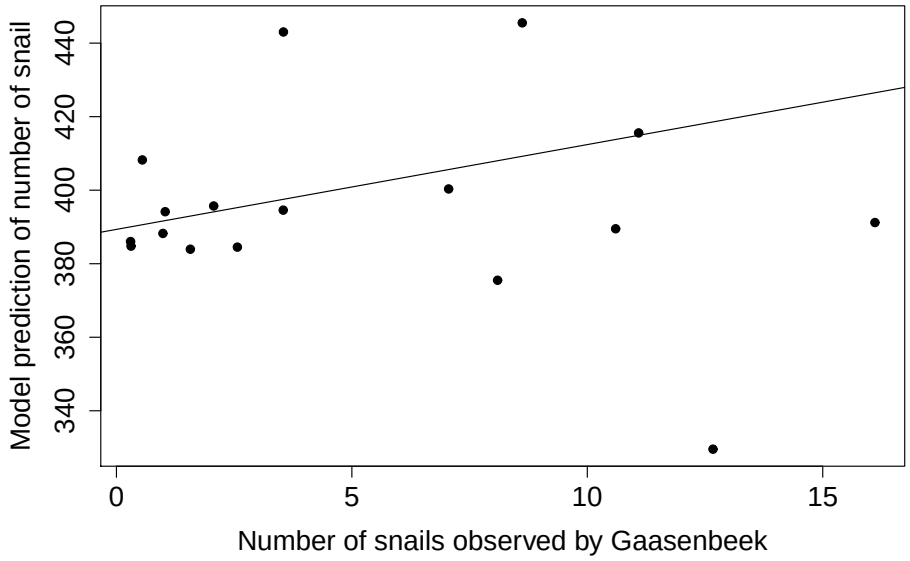


Figure 3.13: The model-predicted snail population plotted against the number of snail observations made by Gaasenbeek 1992 between 1987 and 1989 with linear regression

Relf et al. [2011] studied the presence of *G. truncatula* in Ireland between August 2006 and March 2008. A larger number of snails were found compared to Gaasenbeek et al. [1992]. The largest number of snails found on any one day was 120 in August 2007 (Figure 3.14). There appear to be two peaks in the number of snails found, one early in the year between March and April, and a second later peak in August. At approximately half the time points, fewer than 20 snails are observed (Figure 3.15). The model predictions appear to match the peaks in the number of snails found although the magnitude of the change is less clearly correlated.

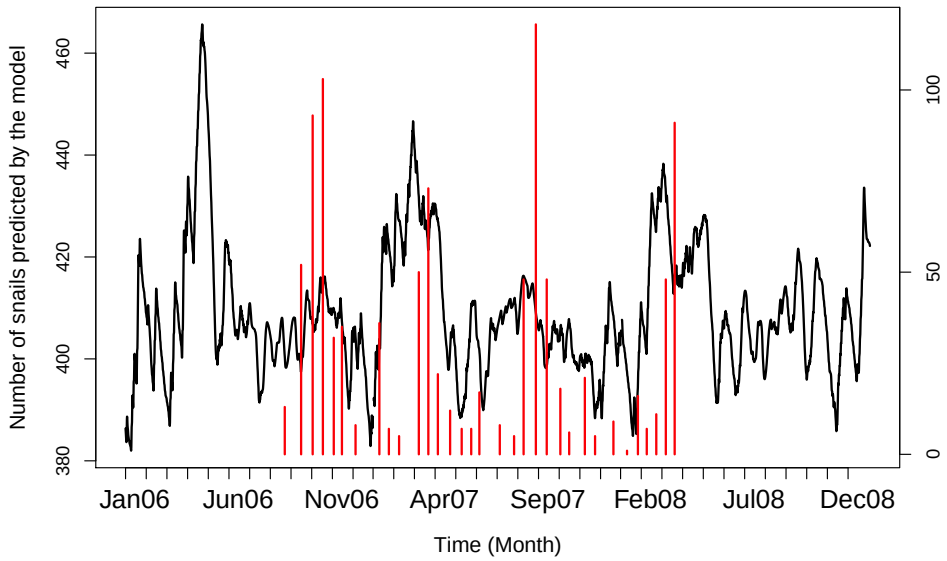


Figure 3.14: The model-predicted snail population (left y-axis, black line) and snail observations made by Relf et. al 2011 in the Republic of Ireland (right y-axis, red bars) between 2006 and 2008

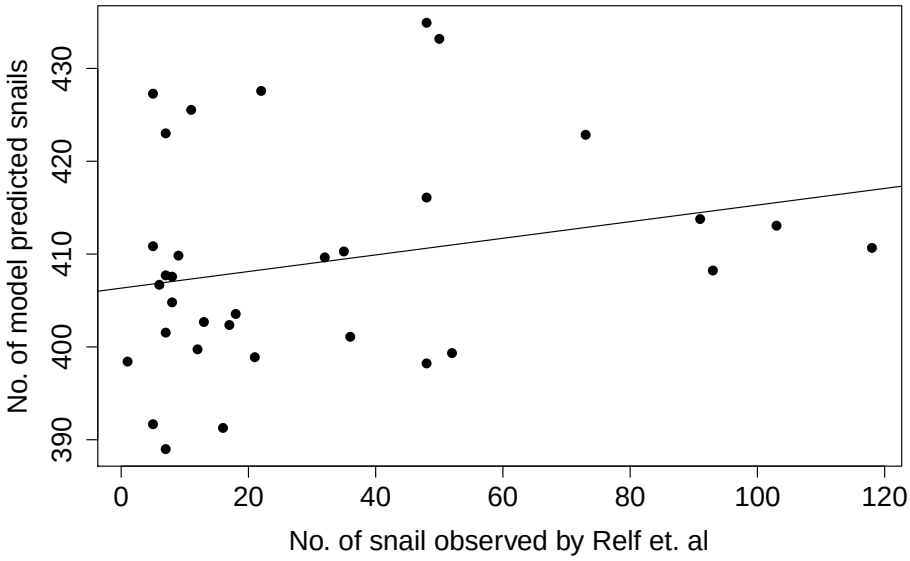


Figure 3.15: The model-predicted snail population plotted against the number of snails observations made by Relf et. al 2011 in the Republic of Ireland between 2006 and 2008, with linear regression line plotted

Charlier et al. [2014a] reported the number of *G. truncatula* snails found per month on two farms in Belgium near Bruges and Zoersel in 2012 (Figure 3.16). Model predictions of the mean monthly number of snails were produced using data from a weather station near both sites. These data were compared to the mean monthly number of snails found on each study farm. The number of snails observed during the study period varied between 10 and over 160 on the study farm in Bruges. There was a weak correlation between the observed number and those predicted on the basis of the model.

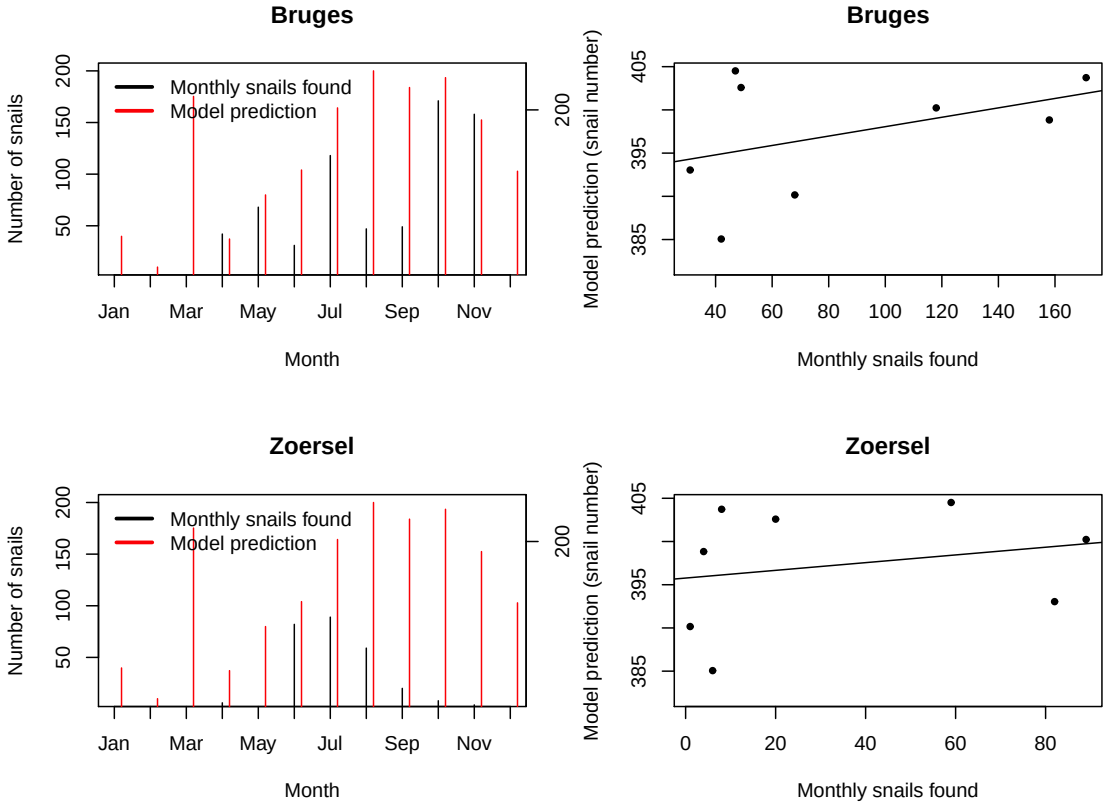


Figure 3.16: Left graphs: The model predicted mean monthly snail population (left y-axis, red bar) and snail observations made by Charlier et. al 2014 (right y-axis, black bars) in Bruges and Zoersel in 2012. Right graphs show the model predictions plotted against the field data collected

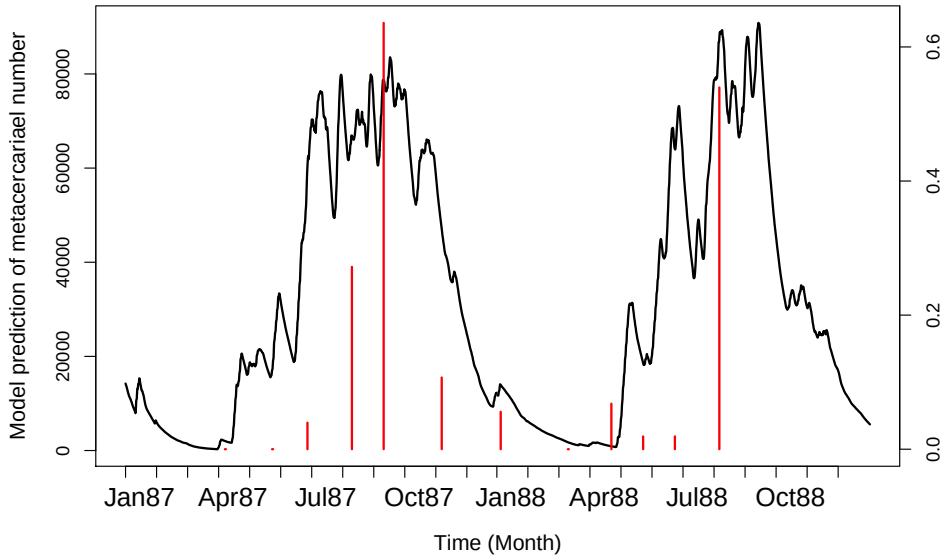


Figure 3.17: The model-predicted metacercarial population (left y-axis, black line) and metacercarial observations made by Gaasenbeek et al. [1992] (right y-axis, red bars) between 1987 and 1988

Figures 3.19 and 3.20 show the correlation between the model predicted number of metacercariae on pasture and the number of metacercariae found in unpublished work by Gaasenbeek between 2004 and 2009. The regression between the observed data temporally and the model predictions (p-value = 0.01, R-squared = 0.29).

The same model prediction data were used to estimate the metacercarial population over time between 1988 and 1989 (Figure 3.17). The prediction of metacercarial numbers for 1988 appears to match the recorded data but the model does not capture the observations from 1989. The metacercarial data recorded between 2004 and 2009 is more extensive (Figure 3.19). Over the 5 years of data, the model prediction of the peak in metacercariae number resembled the recorded data. At the majority of time points, very

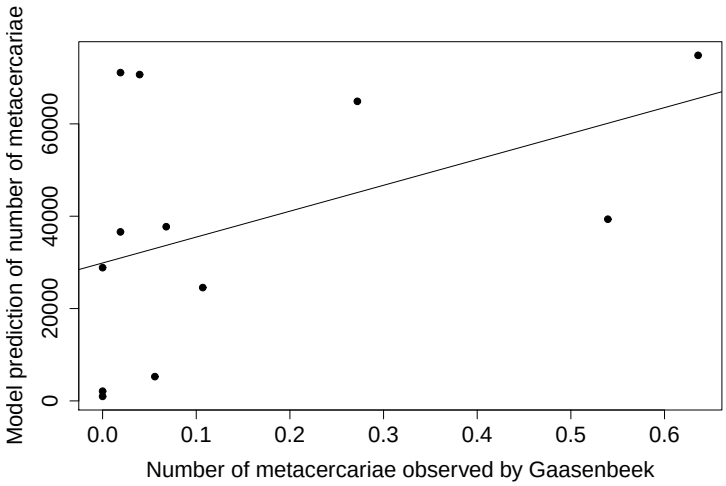


Figure 3.18: The model-predicted metacercarial population against metacercarial observations made by Gaasenbeek et al. [1992] between 1987 and 1988, with linear regression

few metacercarial were found, in most cases fewer than 20. The model prediction of the peak time for metacercarial abundance appears to correlate with time when metacercariae were more abundant in the environment. The metacercariae found by Gaasenbeek are a sample of the total metacercariae that are predicted by the model. There is the possibility of bias and confounding in the data collected, making it difficult to compare the two data sets.

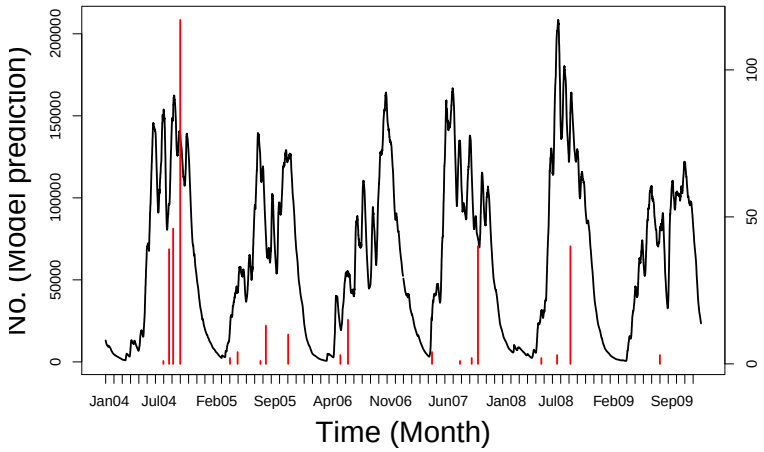


Figure 3.19: The model-predicted metacercarial population (left y-axis, black line) and metacercarial observations (Gaasenbeek Unpublished 2004 - 2009 per comms) (right y-axis, red bars)

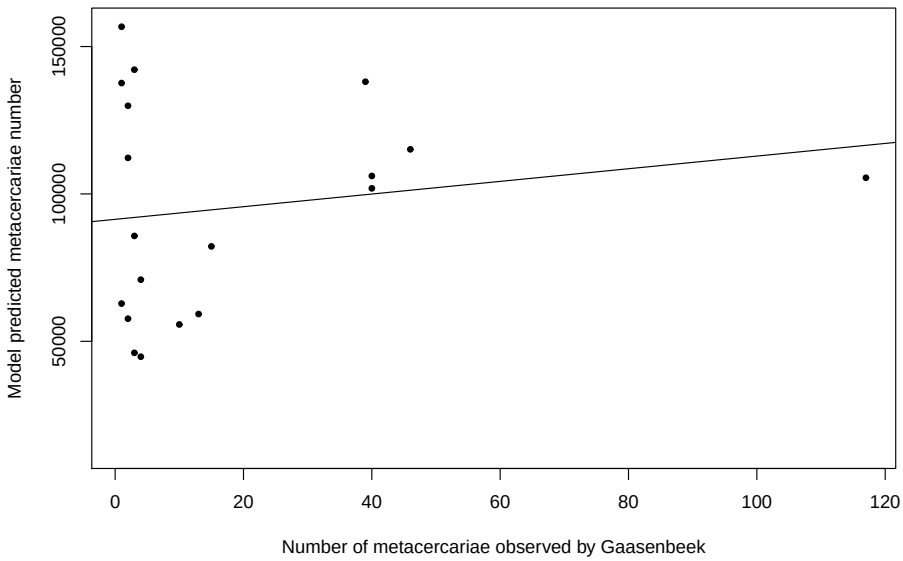


Figure 3.20: The model-predicted metacercarial population against total metacercarial observations by Gaasenbeek Unpublished 2004 - 2009 with linear regression line (per comm)

Figures 3.21, 3.22 and 3.23 show the mean monthly number of metacercariae predicted by the model two months before the recorded VIDA data. Both Scotland and South West England have low numbers of acute fluke cases each month. The most recorded in a single month, between 1978 and 2007, was 4 in South West England and although the maximum number in Scotland was 35, over 90% of months had fewer than 5 cases reported. There were more cases per month reported in Wales over the study period but, in most months, very few acute cases are reported. The regression between the model predictions and the acute fascoloses VIDA data for Wales is significant (p-value = <0.0001, R-squared 0.24). There is less strong statistical evidence to support a correlation between the model predictions and the VIDA data for Scotland (p-value = 0.1, R-squared 0.02) or South West England (p-value = 0.3, R-squared 0.01).

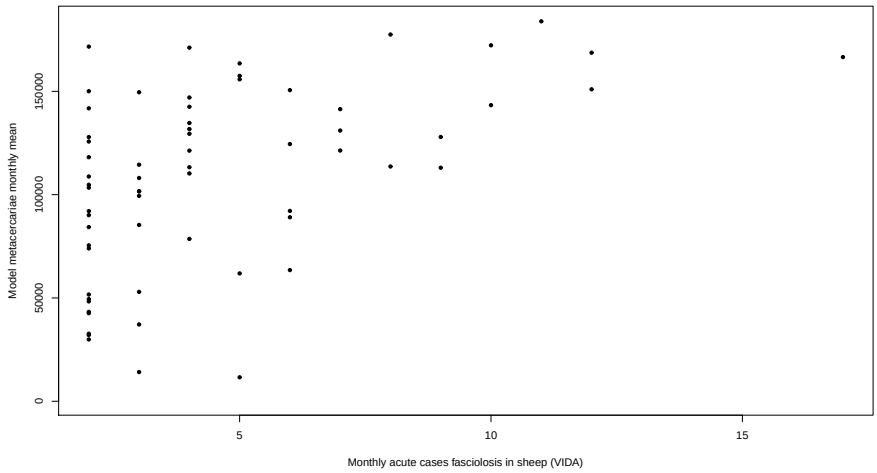


Figure 3.21: Monthly model predictions for Wales between 1978 and 2007 plotted against the number of acute fasciolosis cases reported by VIDA

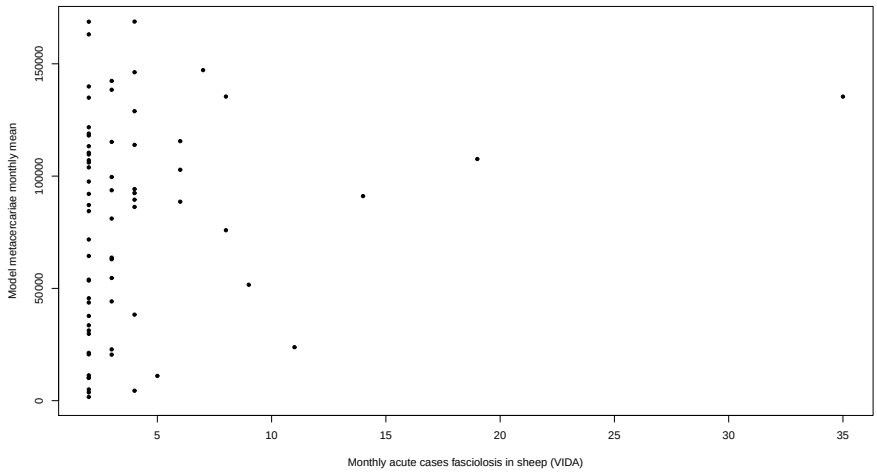


Figure 3.22: Monthly model predictions for Scotland between 1978 and 2007 plotted against the number of acute fasciolosis cases reported by VIDA

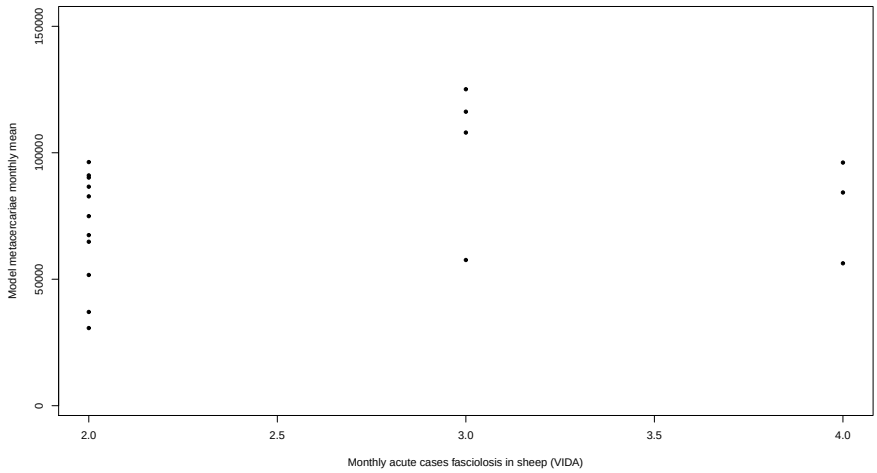


Figure 3.23: Monthly model predictions for South West England between 1978 and 2007 plotted against the number of acute fasciolosis cases reports by VIDA

Ollerenshaw model

In the UK, the Ollerenshaw model is used by the National Disease Information Service [NADIS] to predict the risk of fasciolosis each year. The components of the model are the number of rain days (days with >1mm of rain) in a month, the precipitation in a month (in mm) and the evapotranspiration rate (calculated by the Penman method [Penman, 1948]). In each month an Mt value is calculated, using the formula:

$$Mt = N(R - PE + 5) \tag{3.3}$$

Where Mt = risk score, N = number of rain days, R = precipitation and PE = evapotranspiration rate.

Mt values are calculated for each of the months between May and October. Then these values are summed across these months to produce the predicted annual fasciolosis risk for that year. The summed Mt values are used to categorise the risk as: <300 little or no disease; >300 to 400, occasional losses; >400 to 474, disease prevalent; and Mt >474, serious epidemic [Ollerenshaw and Rowlands, 1959].

To compare the predicted risk from the Ollerenshaw model, to that derived from the differential equation model developed in this thesis, data from the locations in Wales, Scotland and the South West of England used in this chapter, were input into both models. For comparability purposes the differential equation model output was only used for the months May to October in each year. The total predicted number of metacercariae were summed over these months. The graphs for Wales (Figure 3.24), Scotland (Figure 3.25) and the South West of England (Figure 3.26) show the comparison of the summed Mt values generated from the Ollerenshaw model and the total number of metacercariae

predicted by the thesis model, over the same period of May to October for each year from 1997 to 2006.

In general the correlation between the two predictive models appear to be poor. In Wales (Figure 3.24) the two models predict lower risk years in 2003 and 2004 and higher risk in 2005, but in the earlier years there is little concordance. In Scotland (Figure 3.25) the Ollerenshaw model predicts substantial variation in risk year to year, particularly in the period 2001 to 2005, but the differential equation model does not predict this. In the South West of England (Figure 3.26) the predictions of the two model appear to be quite different.

Figure 3.24: Comparison of the Ollerenshaw Mt values to the thesis model metacercariae prediction between 1997 and 2006 in Wales

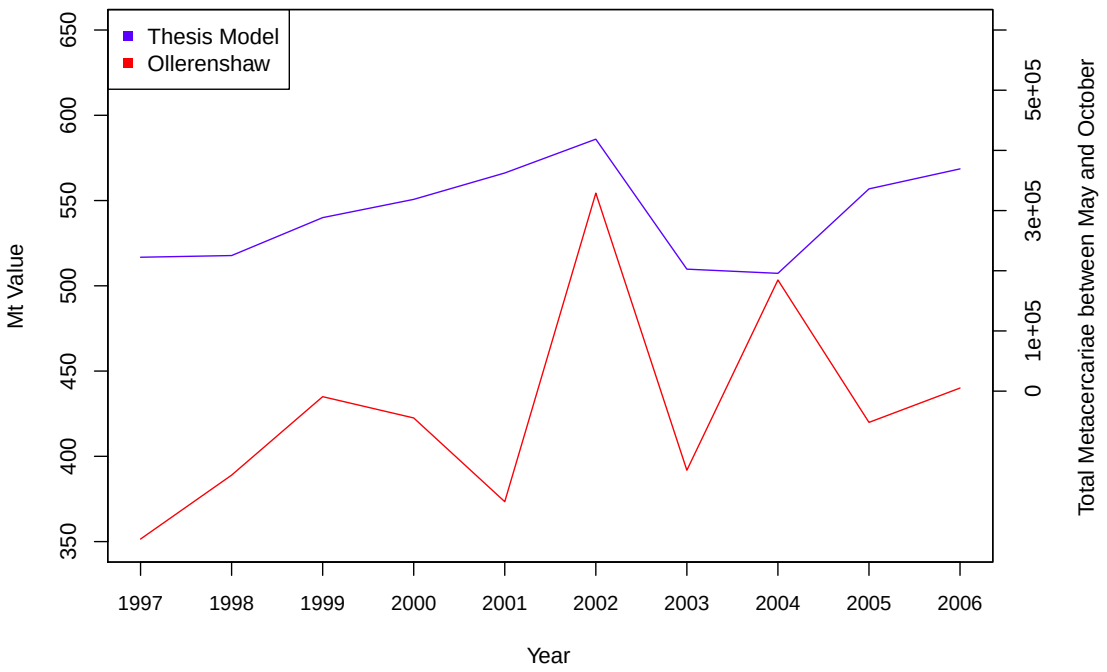


Figure 3.25: Comparison of the Ollerenshaw Mt values to the thesis model metacercariae prediction between 1997 and 2006 in Scotland

Figure 3.26: Comparison of the Ollerenshaw Mt values to the thesis model metacercariae prediction between 1997 and 2006 in South West England

3.4 Discussion

The aim of the mathematical model developed in this thesis was to predict the effect of climate, specifically temperature and rainfall, on the abundance of *F. hepatica* metacercariae in the environment, which could affect the risk posed to grazing sheep and cattle. The model constructed is the first temporally explicit model of *F. hepatica* abundance. Previous models have not focused on the biology of the parasite. Rather, they have used data on infections recorded in sheep and cattle and correlated these with climate data [Ross, 1970]. The aim of these models was to predict when infection levels would be highest, based on climate data in the preceding months, without providing information on when pasture would become dangerous.

The dramatic fluctuations in the snail populations, described in the literature [Shaw and Simms, 1930, Pantelouris, 1963, Stromberg, 1997], are captured by the model. Snail populations are known to have the capacity to explode, and decline rapidly when climatic conditions are less favourable. The younger stages of the snail life cycle appear especially sensitive to fluctuations in climatic conditions. Mature snails appear sturdier and are present in a more year-round pattern. The explosions of the snail population appear to occur especially when the development and / or survival of eggs and young snails becomes favourable.

The model appears to predict realistic patterns of metacercarial presence, with a small spring peak (winter infection, emergence of metacercariae from over-wintering snails), followed by a much larger summer peak (summer infection, arising from eggs which have started to develop in the same year, leading to disease in autumn). The model appears to predict the rise in metacercarial abundance at pasture earlier in the year than perhaps

previously thought. However, metacercariae were found this early in the year in the studies by Gaasenbeek 1992. The summer infection is associated with acute disease in late summer/early autumn, and it may be that the build-up of large fluke burdens in animals accumulates from a time-point earlier in the year than previously thought. Predictions of the emergence of metacercariae clearly facilitate the optimisation of control programmes, notably when to dose animals or remove them from dangerous pasture.

A limitation of the model created in this thesis is the parametrisation of the effects of water on the development and mortality of the fluke and its intermediate host. In the model rainfall influences metacercariae mortality, snail mortality, snail activity and the area of snail habit. This man result in the effect of rainfall being under estimated, the main influence that rainfall exhibits in the model is by increasing of reducing the intensity of pasture contamination by metacercariae where the temperature is the main determinant of the seasonality of the model predictions. The impact of water availability is challenging to study under laboratory conditions and would be challenging to apply to field conditions as there are multiple factors that determine the environmental moisture and these can be very localised.

3.4.1 Constructing the model

There was a large body of published data on the effect of temperature on the development and mortality of *F. hepatica* life stages at temperatures within the range of 10°C to 30°C. At temperatures below 10°C, there was no development or growth of the parasite or of the snail host. At temperatures above 30°C, there is very high mortality of all parasite and snail stages such that, at these temperatures, transmission is unlikely. Therefore, the parametrisation of the model was developed between these temperatures (10°C to 30°C).

At low temperatures, below 10°C, snails will aestivate and development of the parasite within is suspended.

There were limited data available on the development of *G. truncatula* at different temperatures, being confined to data on snail development rates at only four temperatures (Figure 3.6). Consequently, there was uncertainty in the relationship between snail development and temperature. The reasons for the lack of data on snail development may be due, at least in part, to the difficulty in maintaining snail colonies under laboratory conditions, because of their requirement for specific pH, soil type and food source. In addition, the risk of contamination by bacteria and fungi in warm humid conditions makes snail colony maintenance challenging. Noting this lack of available data, the author attempted to set up a snail colony to investigate development and snail egg laying at constant temperatures. *G. truncatula* were gathered from a farm with a known history of disease and brought into a temperature-controlled incubator. However, it was impossible to maintain the colony in the laboratory and, consequently, further data were not generated on the relationship between snail growth and temperature. The ideal experiment would have been to create a small area of controlled pasture in the laboratory, where the temperature and water level could be carefully controlled to observe transmission under more natural conditions than a petri dish in an incubator. However, this was beyond the scope of the research.

3.4.2 Validation

The comparison of the predictions of a mathematical model using field data, that have not been used in the modelling process is, generally, a critical element in the validation of a model. An attempt was made with this model to validate the snail element of the model as well as the final metacercarial stage.

For both the snail and metacercarial validation, some of the variation in the validation data was captured by the model but, largely, validation data were poorly explained by the model. It appears that the the model predicts the timing of metacercarial emergence well but not the scale of the increase. Overall, this results in poor correlation with validation data. It is not possible from the validation data used to determine whether this is due to model failure or due to the type of data used for the validation. The model predicts the total population of snail and metacercariae on the pasture whereas the validation data are based on a small sample of a population, which is strongly influenced by the conditions of the pasture. For example, with regards to validation of the snail part of the model, if it has been dry for several days prior to sampling, then a greatly reduced number of snails will be found as they burrow into soil in dry conditions and thus would generally not be detected using the standard survey methods. Therefore, snails may be present but not found. Indeed, it appears that model predictions over-estimate snail presence. Where snails are found either side of a zero-return field sampling data point, it appears that snails can indeed be present but not found at pasture. It appears that the model may have to include a snail activity parameter. A further confounding factor is that individual investigators vary in their success in finding snails.

Using the VIDA data to validate a model of this type is challenging. It is a limited

dataset with poor regional data, and many years lacking any reported cases [van Dijk et al., 2008]. For Wales, which has the most acute fluke cases, there is a significant correlation between the model and the VIDA data but a very small R- squared value. This suggests that the model is predicting the seasonality of the transmission accurately but due to the difference in the measured outcomes (metacercariae on pasture vs acute cases in sheep), it is not unexpected that the magnitude of the variation in the VIDA data is not strongly correlated. It is also unlikely that, in years where the model predicts high risk and there are no reported cases in the VIDA data, no acute cases of fasciolosis occurred which is one of the limitations of passive surveillance data.

When comparing predictions of risk from the model created in this thesis to the risks predicted by the Ollerenshaw model there was a poor correlation. This may be due to a number of factors; the two models have different primary drivers, for the Ollerenshaw model these are rainfall and evapotranspiration whereas the differential equation model is more strongly influenced by temperature and to some extent rainfall. This is due to the relative lack of quantitative data on the relationship between parasite development and rainfall. The Ollerenshaw model does not directly model the parasite and is, therefore, less adaptable to changing climatic conditions. In addition when applied to areas with significantly different climatic conditions to Anglesey, where is was originally validated, the reliability of its predictions is questionable [Caminade et al., 2015].

When considering the differential equation predictions for metacercariae, it is important to note that the relationship between the number of metacercariae on pasture and risk of fasciolosis is unknown. It is presumed that the greater the number of metacercariae the higher the level of infection in the mammalian hosts. This relationship between the number of metacercariae and risk of disease may not be linear. The differential equation

model predicts the changing risk throughout the year compared to the single seasonal risk of the Ollerenshaw model. If the models were used in combination they could both provide useful data on the transmission of fluke, not only the level of risk, but the timing of the peak of risk as well. As stated previously, there are few data to validate either model and in order to determine which provides the best prediction of the risk of fasciolosis further active surveillance data would need to be obtained.

There are several other possible reasons which could explain the poor correlation found between validation data and model predictions. First, the parameters used in the model are static but, in reality, the variance in responses of individual free-living stages to temperature is large. Future models should explore the effects of parameter stochasticity on model output. Second, egg shedding patterns are kept constant in the model, whereas host egg shedding patterns will be seasonal, and perhaps follow metacercarial abundance patterns time-lagged by approximately 10 weeks. The effects of seasonality in egg shedding patterns should be explored and, ideally, the validation efforts repeated after linking the model described here to a model of the parasitic phase. Third, rainfall varies strongly between localities, quite likely even between neighbouring farms. Temperatures may also vary, for example between neighbouring upland and lowland farms. Therefore, it may not be appropriate to use climate data measured at one location to predict *Fasciola* abundance in another, even neighbouring locations. Indeed, McCann et al. [2010] found that statistical models, containing a large number of climate variables could explain variation in *Fasciola* abundance well at the municipality level, but not at the farm level. These problems are compounded when VIDA data, gathered over a large geographic area, are used. Climate data gathered at a single geographic point are unlikely to give representative model outputs for a whole region. If models like these are to be used as real-time risk

prediction models, they will quite likely either have to run at farm level, or, alternatively, give less precise general predictions for a county or postcode.

The model, as run in this Chapter, therefore certainly has shortcomings but appears to predict the seasonality of fluke epidemiology quite well. Regardless of the precision of exact predictions, it can be used to predict future shifts in seasonality as well as the effect of the timing of anthelmintic drug applications. This will be explored in the next chapter.

Chapter 4

The application of the mathematical model of *F. hepatica* to predict the effect of climate change

4.1 Introduction

There is a widespread consensus that over the rest of this century there is likely to be an increase in the average global temperature due to greenhouse gas (GHG) emissions [Field et al., 2014]. This has led to predictions of changes in the geographic distribution of vector-borne infectious diseases as the distribution of many vectors is limited by temperature conditions [Stromberg, 1997]. This also applies to helminths which undergo part of their life cycle in the environment [O'Connor et al., 2007]. The distribution of fluke is largely determined by the availability of its snail intermediate host. Global temperature change may affect both the geographic distribution of the snails and the abundance of *F. hepatica* [Caminade et al., 2015, Fox et al., 2011]. One of the consequence of global warming is likely to be milder wetter winters and warmer dryer summers in many temperate zones [Field et al., 2014]. Such changes are likely to affect the seasonal abundance of the parasite

as well as disease prevalence. This is especially important in Europe as a major limiting factor for parasite development is low winter temperatures. With milder winters, the epidemiology of infection may change, which may require adjustment of fluke control strategies [Caminade et al., 2015].

Models have been developed to predict the abundance of fluke depending on temperature and rainfall but have typically been based on data from only the part of the year when transmission is known to occur currently [Ollerenshaw and Rowlands, 1959, Ross, 1970]. These models cannot be easily adapted to alterations in the seasonality of infection. The model developed in this thesis overcomes these restrictions by taking account of the effect of temperature on the biological development of the different life stages of the parasite and the snail host throughout the year. It is possible to use our model to predict how the epidemiology of infection might change in the UK and Europe as global warming affects the climatic conditions in these regions. Over the past 100 years, Europe has warmed by a mean of 0.8 °C [Watson et al., 1997]. It is estimated that the average temperature will rise between 1 °C and 3.5 °C by the year 2100 [Field et al., 2014].

Fox et al. [2011], using the Ollerenshaw model and UKCP09 predictions, predicted that fasciolosis risk in the UK is likely to increase by 2050, both geographically and temporally. Fox et al. [2011] stated that there would be more risk in the summer with relatively little change in the winter disease risk. Caminade et al. [2015] conducted similar work for Europe using five global climate models as well as data on cattle densities and found that many areas that were historically suitable for high cattle densities are also high risk areas for fluke infections. Under high GHG emissions scenarios, the risk of fasciolosis was significantly increased in Northern Europe, although under moderate emissions scenarios, the increase is less marked [Caminade et al., 2015].

Parasites generally have a range of temperatures within which they are able to develop. For *F. hepatica*, this is between 10 °C and 30 °C. Below 10 °C, development is suspended and above 30 °C, there is very high parasite mortality. For some stages of the *F. hepatica* life cycle, there is a non-linear relationship between temperature and the rate of development, such that a small increase in temperature can cause a large increase in development rate. For much of North West Europe, the limiting variable for development of the parasite is low temperatures for much of the year [Caminade et al., 2015]. With climate change predicted to increase the average global temperature by several °C over the next 100 years, this will mean that a larger proportion of the year will be suitable for parasite development. The average temperature increase is unlikely to be sufficient to rise above the critical temperature of 30 °C and result in increased parasite mortality [van Dijk and Morgan, 2008].

Changes in the severity of infection and seasonality of disease will necessitate alterations in the management practices currently used on farms to control *F. hepatica* transmission. One method to control fasciolosis in animals is to try to interrupt its life cycle by reducing the contact between the intermediate host and the parasite. This could be achieved by several methods, including treating animals to stop the shedding of eggs for a period of time, or restricting animal access to areas of snail habitat when there are snails available for infection. Here, we have deployed the developed model to 1) explore future seasonal abundance of *F. hepatica* under different climate change scenarios, 2) to investigate how to maximise the effect of preventative anthelmintic treatments by optimising treatment timing and 3) to explore how preventative treatments will have to be timed in future.

4.2 Methods

4.2.1 The effect of climate change on metacercarial abundance

The three geographic locations in the United Kingdom, also used in Chapter 3, were also selected to provide model estimates of the 30 year mean monthly metacercarial abundance between 1978 and 2007 using recorded temperature and rainfall data. In addition, predictions were made of metacercarial abundance in the same areas between 2017 and 2047, based on temperature and rainfall projections under two different climate models from the Representative Concentration Pathways (RCP) emission scenario database [Field et al., 2014]. The two scenarios were, a medium emission scenario (RCP45) and a high emissions scenario (RCP85) for Wales, Scotland and South West England. Comparison was made of the predicted historic abundance patterns with the future predictions under the two climate change models. These have been used to assess whether climate change will affect the seasonal pattern and levels of infection.

4.2.2 Effect of host treatment with an appropriate anthelmintic on metacercarial abundance

The same three UK geographic locations were also used to estimate the effect of different host treatment strategies on the metacercarial population, using both the historic and future predictions of metacercarial abundance. The model assumes animals are grazing on pasture all year round and, therefore, parasite eggs are constantly being deposited into the environment by infected animals, at a constant rate. To understand the effect that treatment of animals in the spring has on the number of metacercariae seen later in the year, two treatments types were considered: Treatment 1. Treatment with triclabendazole is effective against flukes of all ages within infected animals unless there is

resistance. Hence, after dosing, assuming no anthelmintic resistance is present, animals, even if grazing contaminated pasture from which they can become re-infected, will not be shedding eggs for 8 - 12 weeks [Boray, 1969]. Therefore, triclabendazole treatment was modelled as a 12-week withhold of eggs being added to the model. After 12 weeks, egg shedding resumed. Treatment 2. Treatment with nitroxinyl and closantel only kill flukes older than 6 weeks, resulting in only 6 weeks of interruption in egg shedding, as there will be surviving immature fluke within the host animal which will shed eggs when reaching maturity in 5-12 weeks [MacKintosh and Brophy, 2012].

The model was used to investigate the effect of these treatments applied to animals in different months of the year to determine in which month treatment had the greatest impact on metacercarial numbers in that grazing season. Scenarios involving treatment in Feb, march, April or May were all considered. This was done for both the historic and future predicted data to determine whether a warming scenario would impact on optimal treatment strategies.

4.2.3 Statistical methods

The monthly predicted mean metacercarial number for the 30 years of historic data (between 1978 and 2007) and 30 years of predicted future data (between 2017 and 2047) were bootstrapped using 10,000 replicates, and the mean and 95% confidence limits for each month under the various climate and treatment programmes calculated. Mann Whitney U tests were performed on the mean number of predicted metacercariae for the months of August to October as these months account for 50% of the total yearly metacercariae to analysed in order to asses the predicted impact of treatments administered during the specified months.

4.3 Results

4.3.1 Effect of climate change on metacercarial abundance

Figure 4.1 shows model prediction for the yearly mean number of metacercariae predicted between 1978 and 2047. Between 1978 and 2007, the model predicts that the annual mean number of metacercariae is relativity stable between 50,000 and 75,000, with slightly higher numbers seen in Wales. The predicted mean yearly metacercarial numbers are higher than the historic predictions, ranging between 100,000 and 150,000.

The seasonality in metacercarial numbers when comparing the future predictions with the historic predictions is similar, with large numbers of metacercariae seen in the autumn, especially between August and October, and lower numbers predicted between January and May. Figure 4.2 shows the monthly mean predicted number of metacercariae and 95% confidence intervals for the 30 years 1978 - 2007 and for 2017 - 2047 under two climate change scenarios. Although the seasonality is similar, the model predictions for the future climate change scenarios are for significantly higher metacercariae numbers throughout the year, with a far higher peak in the autumn. In addition, there are higher numbers of metacercariae seen in December and January suggesting that there is increased over-winter survival of metacercariae.

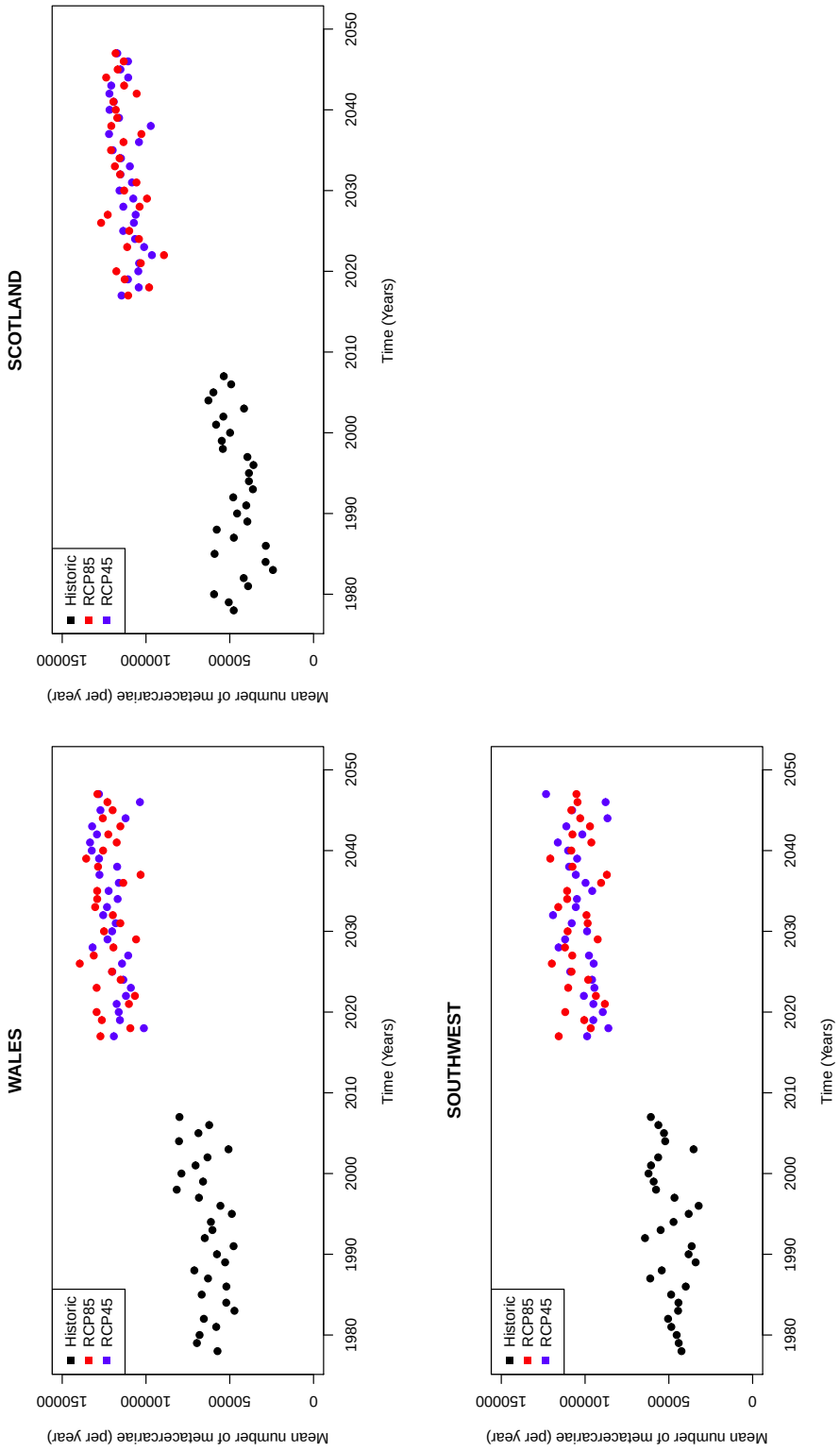


Figure 4.1: Predicted mean number of metacercariae per year under historic 1978 - 2007 (black) and 2 future climate change scenarios 2017 - 2047 (RCP45 (Blue) and RCP85 (Red))

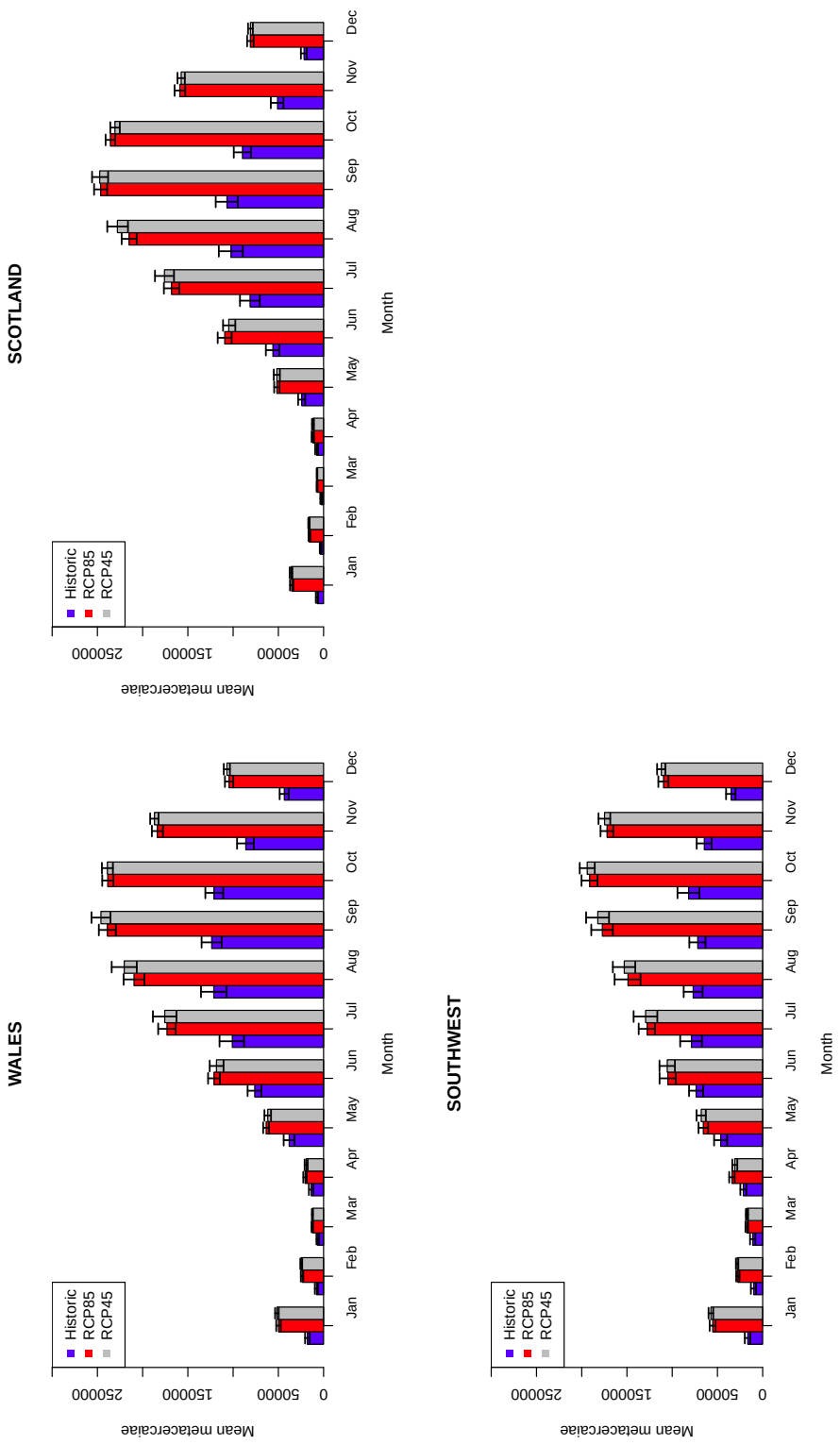


Figure 4.2: Predicted mean number of metacercariae by month for 1978 - 2007 and under 2 climate change scenarios (RCP45 and RCP85) for 2017 - 2047

4.3.2 Effect of host treatment with an appropriate anthelmintic on metacercarial abundance

Figure 4.3 shows the predicted effect of triclabendazole treatment of animals in February, March, April or May between 1978 and 2007. The impact of treatment is greatest during the months of July to December if treatment is administered in May rather than earlier in the year. As expected, metacercarial numbers in the months of January to June are not affected by any of the treatment strategies. Figure 4.4 shows the predicted effect of Nitroxynil/Closantel treatment of animals in February, March, April or May between 1978 and 2007. Treatment with these compounds alone does not appear to affect the number of metacercariae later in the year to a significant extent.

Figures 4.5 and 4.6 show predictions in a similar manner as in Figure 4.3 but for the period of 2017 - 2047 under climate change scenarios RCP85 and RCP45, respectively. The predicted metacercarial numbers by month are higher than for the historic series but the general patterns are similar, showing the greatest reduction in the period July to December if treatment with triclabendazole is administered in May.

Figures 4.7 and 4.8 show predictions in a similar manner for the period of 2017 - 2047 but for treatment with nitroxynil/closantel. Compared with the historic model predicted data (Figure 4.7), the effect of treatments are greater and treatment in May reduces metacercarial numbers to a greater extent in the period July to December than treatments given in February, March or April.

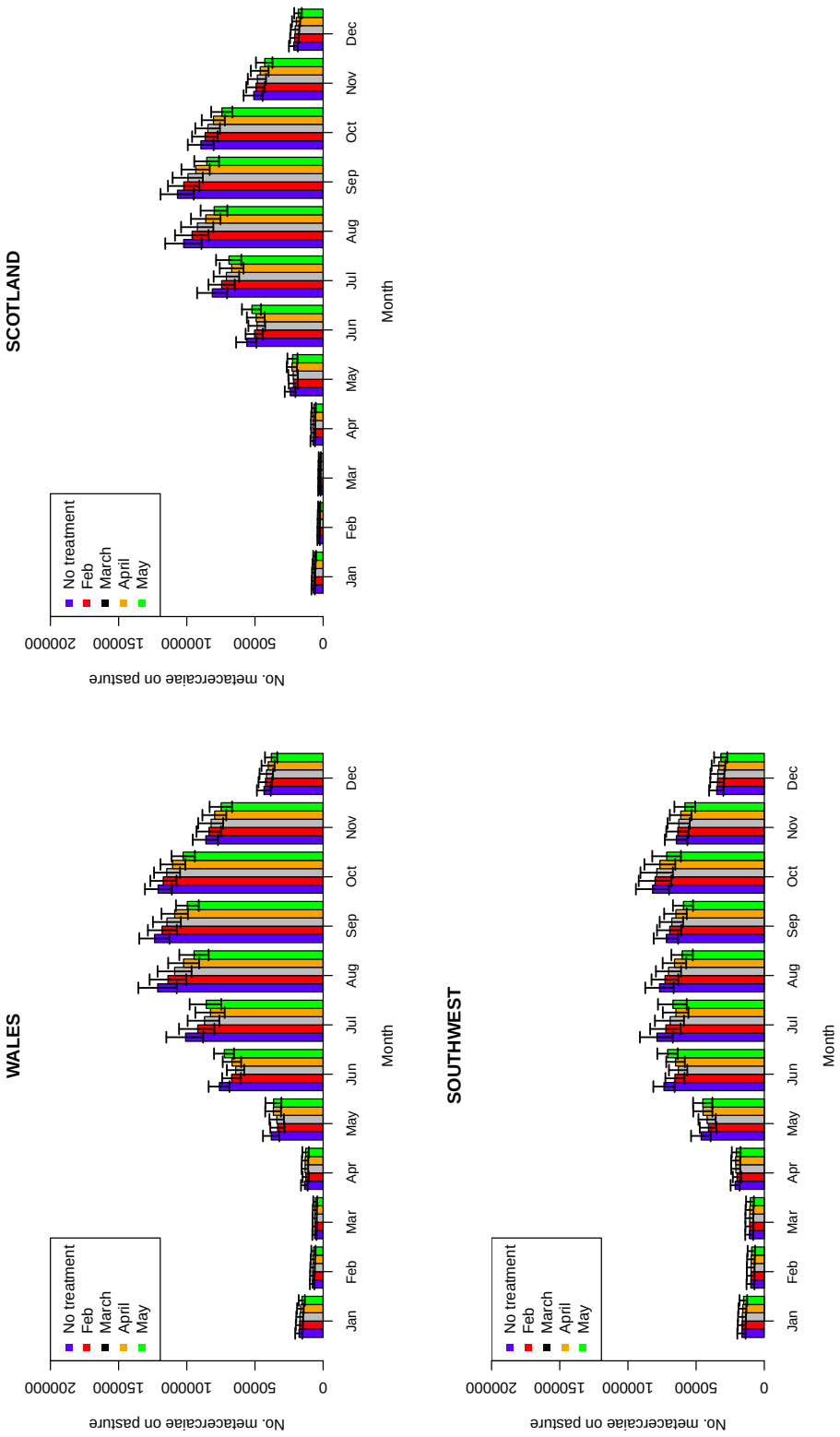


Figure 4.3: Predicted number of metacercariae in each month, averaged over 1978 - 2007, and under different climate scenarios if triclabendazole is used

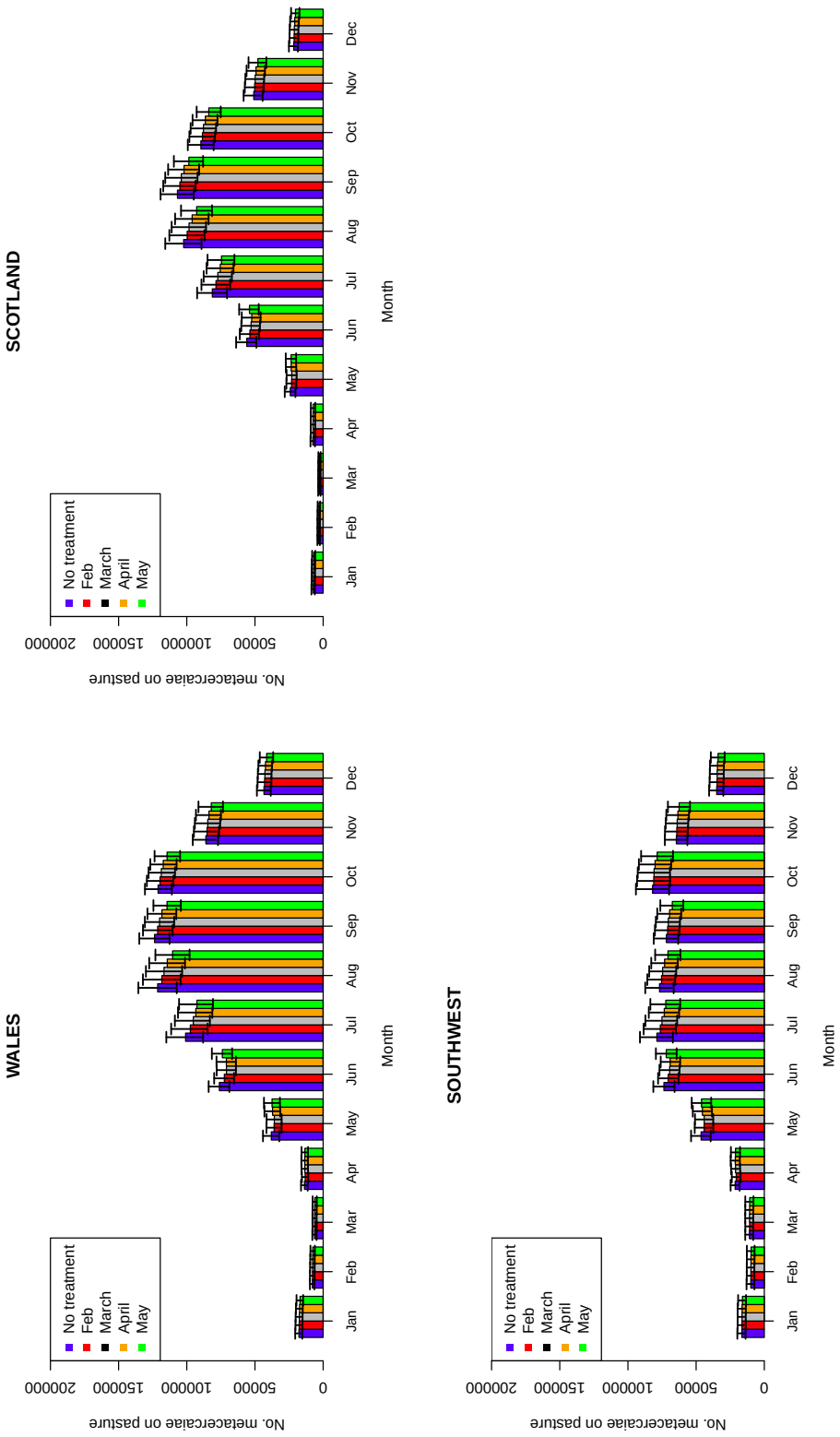


Figure 4.4: Predicted number of metacercariae in each month, averaged over 1978 - 2007, and under different climate scenarios if Nitroxynil/Closantel is used

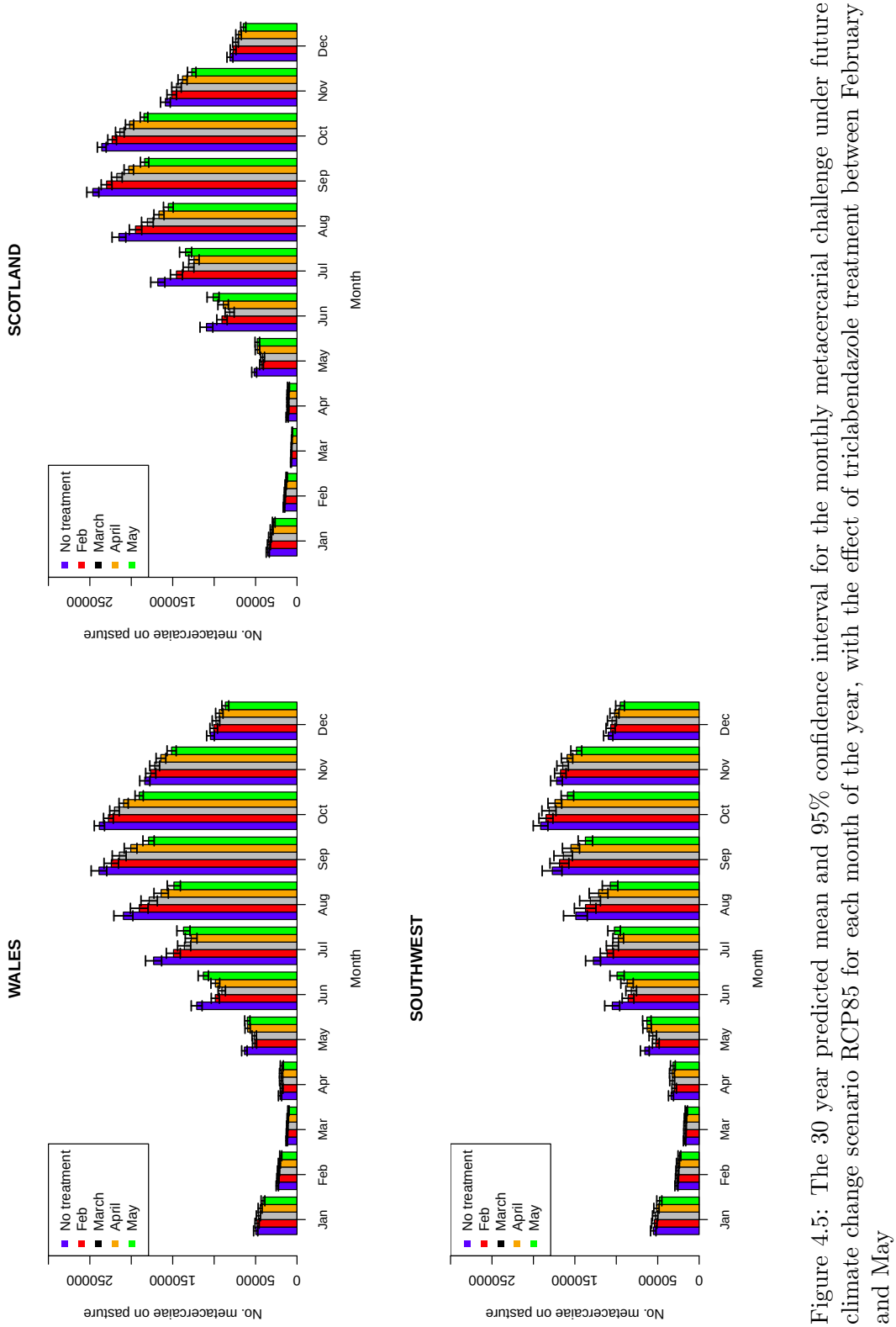


Figure 4.5: The 30 year predicted mean and 95% confidence interval for the monthly metacercarial challenge under future climate change scenario RCP85 for each month of the year, with the effect of triclabendazole treatment between February and May

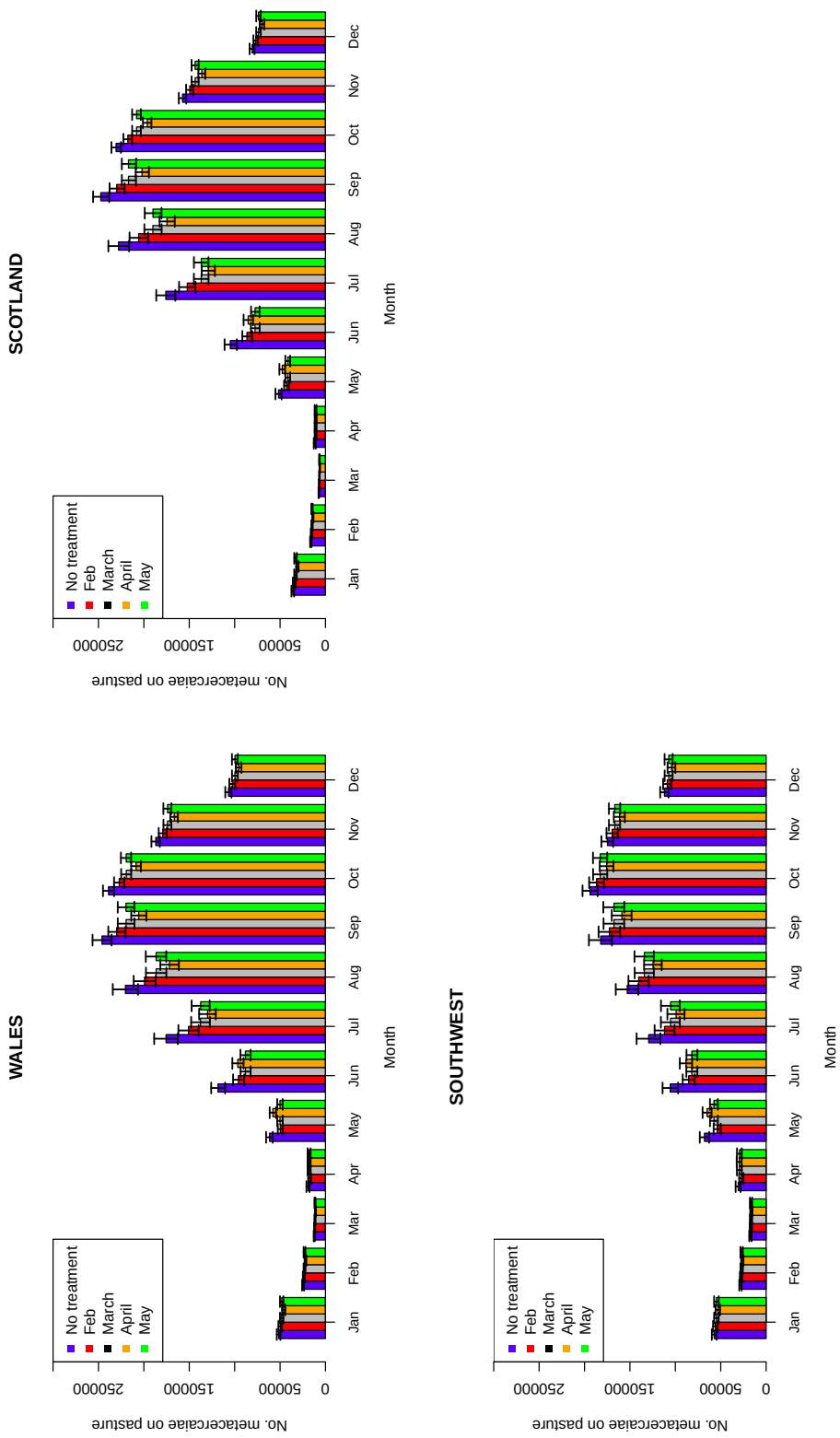


Figure 4.6: The 30 year predicted mean and 95% confidence intervals for the monthly metacercarial challenge under future climate change scenario RCP45 for each month of the year, with the effect of triclabendazole treatment between February and May

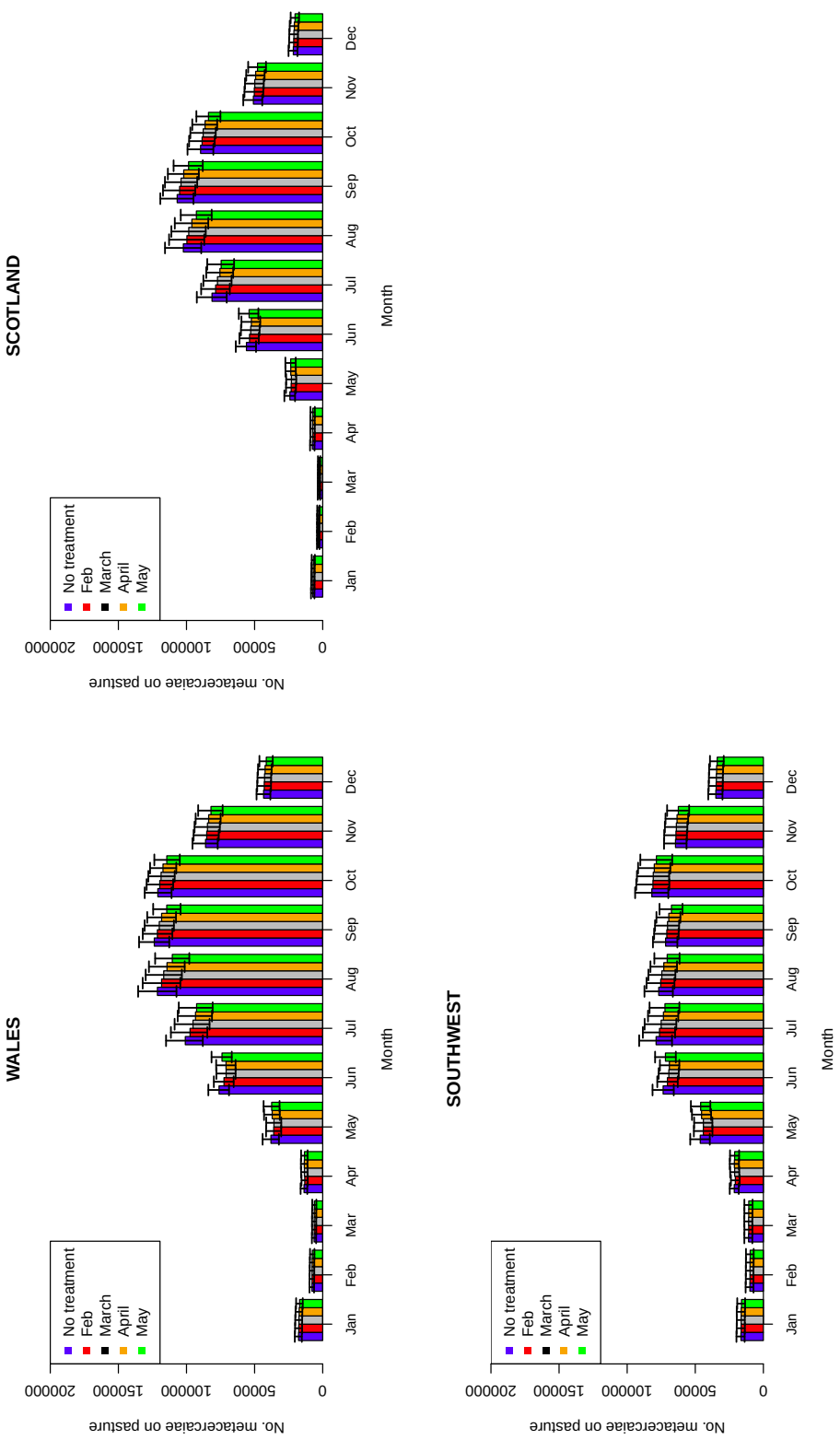


Figure 4.7: The 30 year predicted mean and 95% confidence intervals for the monthly metacercarial challenge under future climate change scenario RCP85 for each month of the year, with the effect of Nitroxynil / Closantel treatment between February and May

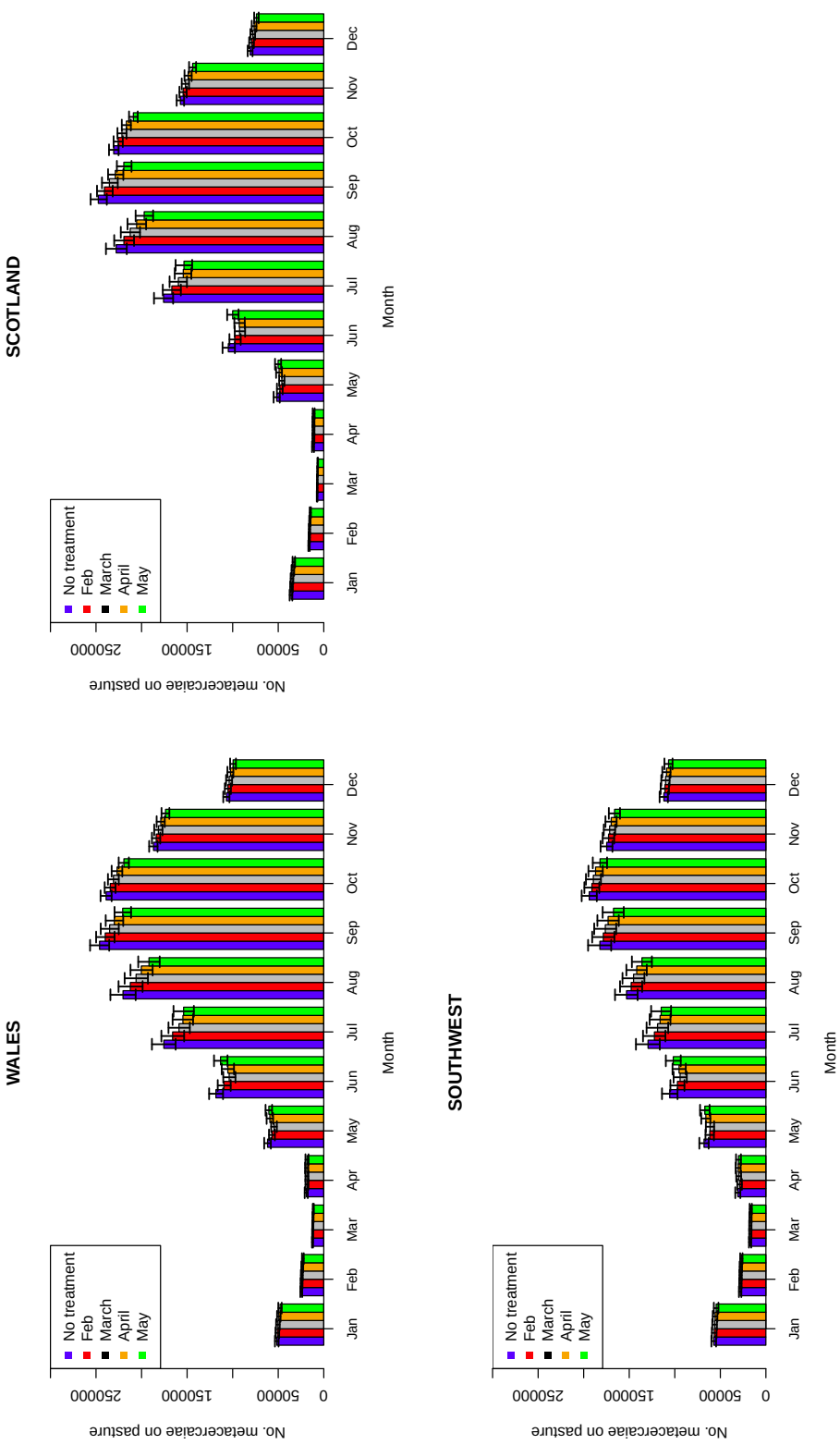


Figure 4.8: The 30 year mean and 95% confidence intervals for the monthly metacercarial challenge under future climate change scenario RCP45 for each month of the year, with the effect of Nitroxynil / Closantel treatment between February and May

Table 4.1: Results from Mann-Whitney U test comparing, no treatment with, treatment with triclabendazole, in different months on the number of metacercariae seen in August to October

Location	Month of triclabendazole treatment			
	February (p-value)	March (p-value)	April (p-value)	May (p-value)
Wales (Historic)	0.4	0.2	0.02	0.0003
Scotland (Historic)	0.5	0.3	0.08	0.006
South West England (Historic)	0.5	0.3	0.13	0.02
Wales (RCP85)	0.008	<0.001	<0.001	<0.001
Scotland (RCP85)	<0.001	<0.001	<0.001	<0.001
South West England (RCP85)	0.1	0.02	<0.001	<0.001
Wales (RCP45)	0.006	<0.001	<0.001	<0.001
Scotland (RCP45)	<0.001	<0.001	<0.001	<0.001
South West England (RCP45)	0.1	0.01	<0.001	<0.001

Model predictions (Table 4.2) suggest that treatment in February and March does not significantly affect metacercarial numbers, although treatment in April and May does reduce the subsequent metacercariae numbers seen in some regions. In the future climate change scenarios, model predictions of the effect of triclabendazole treatment in all months between February and May suggested a significant reduction (p-value =<0.001) in metacercarial numbers in August to October, apart from treatment in February in South West England, which does not significantly reduce metacercarial numbers (p-value =0.1).

Table 4.2: Results from Mann-Whitney U tests comparing, no treatment with, treatment with Nitroxynil /Closantel, in different months on the number of metacercariae seen in August to October (DF= 29)

Location	Month of Nitroxyn/Closantel treatment			
	February (p-value)	March (p-value)	April (p-value)	May (p-value)
Wales (Historic)	0.7	0.5	0.4	0.2
Scotland (Historic)	0.6	0.6	0.6	0.3
South West England (Historic)	0.7	0.6	0.5	0.3
Wales (RCP85)	0.3	0.06	0.005	<0.001
Scotland (RCP85)	0.1	0.009	<0.001	<0.001
South West England (RCP85)	0.4	0.3	0.1	0.02
Wales (RCP45)	0.3	0.06	0.008	<0.001
Scotland (RCP45)	0.1	0.01	<0.001	<0.001
South West England (RCP45)	0.5	0.3	0.2	0.02

4.4 Discussion

For the RPC45 and RPC85 climate change emission scenarios, the mathematical model predicts that there will be an increase in the number of metacercariae on pasture throughout the year in Wales, Scotland and South West England. For Wales and Scotland, the peak in metacercarial numbers is in October, both in the historic data and the predicted future data, although, there are far greater numbers of predicted metacercariae in the future data. South West England shows a different pattern in the number of metacercariae throughout the year. In the historic data, there is no single peak in infection but rather a gradual increase that plateaus between June and November. In the future predicted data, there is an increase in the number of metacercariae in all months but there is also a more defined peak in metacercarial numbers towards October. For all three locations, there ap-

pears to be an increase in overwinter survival of metacercariae with higher metacercariae numbers in the winter months, this could result in the higher levels of infection seen in spring. In Wales and Scotland, approximately 50% of the metacercariae are present between August to October and in South West England, this time period accounts for approximately 40% of metacercarial abundance.

The current level of metacercariae predicted during the historic peak (1978 - 2007) in October will be seen in June under projected climate change conditions and remain above this level until December. This systematic predicted increase in metacercarial numbers could result in more disease transmission throughout the year and more acute disease occurring not only the autumn but early summer as well. Consideration of different treatment strategies indicates that under both historic and future climate change scenarios, treatment of animals in May produces greater reductions in metacercarial abundance than treatment in February, March or April. Treatment with an adulticide before animals are turned out onto pasture would give the same duration of no eggs shedding as triclabendazole. This would reduce the usage of triclabendazole which parasites have begun to show resistance to [MacKintosh and Brophy, 2012] and would clear any fluke present in the animals as juveniles are unlikely to be present after housing.

Chapter 5

Concluding Discussion

The primary objective of this thesis was to create a model for the free-living stages of *F. hepatica*, driven by climatic variables so that it was possible to predict changes in the abundance of the parasite according to temperature and rainfall patterns. This is the first model for *F. hepatica* to parametrise the relationship between climatic conditions and its life cycle of both the parasite and the snail host. For the first time, peak (danger) periods of metacercarial abundance can now be predicted. In addition, the model can also take into account changes in farm management practices, such as alternative treatment strategies which have not been included in previous models. The model was developed using parasite growth and mortality rates driven by temperature and rainfall, using data from published papers, as well as experimental work conducted as part of this thesis.

Some of the challenges encountered while developing this model were regarding the availability and reliability of data on *F. hepatica* egg and metacercarial survival and development, as well as on snail growth and mortality. Therefore, the work presented here has identified a need for "old fashioned" experiments, studying the ecology of the parasite, and its snail intermediate host, both in incubators and on pasture. There is also great value in "basic" validation data collection work, counting snails and parasitic stages, perhaps

crawling pasture on hands and knees like the early workers. There would be great merit in the establishment of internationally agreed protocols to collect such data, so that their quality can be assessed and the data can be interpreted. Most of the available literature described experiments and models created between 1960 and 1990, when climate was considered relatively stable. It was not until the 1980s that climate change first became a public and political issue, which meant that previous models did not focus on the rapid changes in temperature and rainfall we are seeing and will continue to observe in the coming years [Field et al., 2014].

Many authors encountered similar limitations in the data available on incidence and prevalence of fasciolosis in livestock, and had to limit their findings to data obtained directly from post mortem examinations [Ollerenshaw and Rowlands, 1959], or abattoir inspection [Goodall et al., 1991]. Smith [2011] emphasises the impact data from specific farms could have on a model rather than using regional data, which could result in inaccurate outcomes when extrapolated to wider areas. This specificity applies to both parasite-host and climatic variables. Field data also present some difficulties, year-to-year or even season-to-season, climate variations could result in over or underestimating parasite burden (Meek 1981). Other difficulties include the collection of field data, as many stages of the parasite life cycle are challenging to sample or the selection of sampling methods which focus on taking samples that are easier to obtain but may not be representative of an area or region [Smith, 2011].

Incorporating too much complexity into a model can result in less accurate predictions [Ollerenshaw, 1966], so there is a fine line between too many variables included and an over-simplification of the model. The complex life cycle of the parasite also presented a challenge when transforming biological processes into differential equations, with some

stages having to be removed or omitted from the model due to short life spans in the environment. For example, the miracidial and cercarial stages were originally included in the model but were later removed after their effects on the outcome of the model predictions proved minimal. These decisions were based on previous results by Al-Habbib [1974], who reported that survival times of these stages (miracidia and cercariae) were less than 24 hours, one day showed little to no influence on model outcome. Data on the development and mortality of the parasite eggs was abundant and reliable, however, the same cannot be said for the intermediate snail host, where the effect of temperature changes on the survival in the environment has been scarcely reported [Nice and Wilson, 1974]. This lack of available data made the validation of the model a difficult process, with some of the data regarding snail abundance in the environment showing a poor relationship between the number of snails observed by Gaasenbeek and the predictions made by the model. However, it is important to mention that the study conducted by Gaasenbeek was done in the Netherlands with data from 1987 to 1989 and this model was built using data gathered in the UK. If there is variation in the response of different isolates to environmental conditions, this could lead to an inaccurate prediction of risk. Temperature and moisture are two of the primary drivers of the population dynamics of *G. truncatula* and maturation of *F. hepatica*, therefore, good data on both have to be available [Relf et al., 2011]. However, the existence of micro-climates might also have an impact on the infection rates seen in animals [Gaasenbeek et al., 1992].

As part of the experimental work conducted for the thesis, attempts were made to establish a snail colony to study the effects of temperature and water availability on growth and mortality. Unfortunately, these attempts were unsuccessful and it did not prove possible to maintain the colony. The reasons for this failure are unclear, although there

were issues with the growth of algae in the laboratory and fungal contamination of the snail habitats within the incubators. Such data would have been useful in the parametrisation of the model. Despite this, even if a colony had been successfully maintained, it is unlikely that parametrisation of the model based on these data would have perfectly replicated snail behaviour under field conditions. In the experimental work, it would have been very difficult to take account of the many confounding factors, such as food availability, the impact of predators and other parasites and other environmental factors.

The model outcomes for climate data predicted for the future, compared to historic climate data, showed an overall increase in the predicted number of metacercariae on pasture throughout the year but with a similar seasonal pattern. Shaka and Nansen [1979] showed that cold wet environments allowed better survival rates of metacercariae at pasture, which would contradict the forecast of this model, however, this prediction could be due to accelerated development and shedding of the parasite rather than due to an increase in survival rates. The downside of pasture studies is that, when metacercariae are counted, it is not know whether these are surviving, older metacercariae or newly developed, freshly shed ones. This agrees with Al-Habbib [1974] regarding increased development and shedding under warmer conditions.

The data on parasite abundance were obtained via the passive surveillance of the APHA and published in VIDA reports between 1978 and 2007. In the case of Wales a good relationship with the model predictions was observed, while the South West of England and Scotland showed a relatively poor relationship. These could be due to better reporting practices in Wales, than in the rest of the UK and not necessarily because of a higher incidence of cases. These data have considerable limitations as, in areas where fluke is endemic, farmers are likely to treat animals for fasciolosis without submitting animals for

diagnosis or just submitting animals for initial diagnosis only and not submitting further cases as they occur. It is difficult to determine from the validation data whether the lack of correlation between the model and observed data reported in Chapter 3 is due to a failure of the model to accurately characterise the populations, or due to the unsuitability of the observational data for model validation.

This model facilitates a better understanding of the risk on metacercariae of pasture when taking into account current climate change trends and possible modification to present day farm management practices, unlike previous models which tended to account for a more stable climate and consistent in farming practices. This model also showed critical timing during which interruption of parasite development could result in a significant decrease in metacercarial populations on pasture and, therefore, less risk of infection for the definitive host. This implies a potential reduction in the production loss when appropriate control measures are applied during these times. This model also showed the critical stage of parasite development which, if interrupted, could result in a significant decrease in metacercarial population on pasture and, therefore, less risk of infection for the definitive host. This means a potential reduction in the production loss when appropriate control measures are applied during these times of year.

Vaccination is currently unavailable but under development, focusing on reduction of adult fluke burden in livestock [Toet et al., 2014]. Depending on the duration of vaccine protection, according to the outcome of this model, the best time to prevent parasites developing into adults and shedding fluke eggs would be spring. The model also shows that, together with vaccination, a decrease in the risk of infection could be achieved by limiting access of livestock to areas where the intermediates snail host is present during the same spring period. This would not only limit the shedding of fluke eggs into the

environment but would also reduce the number of metacercariae ingested by the livestock.

In experiments, metacercariae were exposed to varying temperature conditions and different levels of water availability. The survival of the metacercariae from different geographic locations was found to vary. The Welsh isolate presented a lower mortality rate at temperatures between 15 °C and 25 °C than the isolate of Italian origin. Higher mortality of the Italian isolate was also observed when the metacercariae were exposed to water stress. This has been a limited study into metacercarial survival, so the observed results may be unrelated to the parasite region of origin, and may instead be due to other confounding factors, for example, that the metacercariae from Italy were produced using snails of Welsh origin. Due to the potential impact of such factors on model predictions, it would be of value for there to be greater exploration of the resilience of metacercarial isolates from geographic regions as investigated by Fairweather [2011]. Until such further studies are carried out on a wide scale, caution should be exercised in applying a model based on isolates in one region to regions where other isolates dominate.

All of the data used to develop the model were based on work conducted on isolates from the UK and Northern Europe. This potentially limits the application of the model to a wider area, outside of Northern Europe, particularly without further data on the differences in survival and development of the metacercarial isolates in other geographic areas. If such data became available, there would be scope to use this model as a framework, by altering individual parameters in the model to be appropriate for local conditions. To expand on the known relationship of parasite development with temperature and water availability, it could be informative to study parasites from different regions from egg development under different conditions through to the survival of metacercariae to observe whether there was any differential effects on the parasite. Although this was not

possible in the work conducted for this thesis, uncovering if selection pressures applied to the parasite eggs has an influence on the subsequent survival of metacercariae would be informative.

Current control based on routine treatment rather than prevention have already led to antihelmintic resistance to triclabendazole [Álvarez Sánchez et al., 2006], which could prove challenging for future control of fluke. However, changing the focus from treatment to prevention, based on targeting vulnerable stages of the parasite, could increase the chance of better control of transmission and infection of *F. hepatica*. Using the model to predict the impact of different treatment strategies indicated that treating animals with a single dose of triclabendazole, would have been successful in reducing metacercarial numbers historically in Wales and Scotland, where there is a defined peak in metacercarial numbers in the autumn. In contrast, this single treatment approach in the South West of England is predicted to be less effective. This is due to the longer period of suitable conditions for parasite egg development, resulting in a longer period needed to avoid pasture contamination. For future climate change scenarios, the timing of treatment will be even more important, with far higher numbers of metacercariae observed. Treatment at the right time, which the modelling suggests is in May, appears to significantly decrease metacercarial numbers during the August to October peak.

Several authors have predicted that *F. hepatica* will increase under future climate conditions [Fox et al., 2011, Caminade et al., 2015]. The models used to make these predictions were originally developed and validated using data from a single sheep farm on Anglesey and were not originally designed to function under conditions of climate change. Although this model has proved powerful in predicting fasciolosis risk, it is limited as it cannot be interrogated to examine the effect of changes in seasonality or farm management practices.

The Ollerenshaw model and the thesis model for Wales showed a similar pattern changing Mt risk with number of metacercariae predicted by the differential equation model. The correlation was poor when comparing Scotland and the South West of England. These two models were designed to function in very different manners and on different time scales, the Ollerenshaw model predicts seasonal risk whereas the thesis model predicts daily risk and can model the peak in risk for the year. Overall, this work has highlighted the lack of reliable data on *F. hepatica* and its host as well as its environmental life cycle stages. Likewise, developing this model also showed the relatively low flexibility of previous models to include changing farm management practise and changing climate conditions which can be critical for increased risk of infection and transmission as previously discussed.

The predictions obtained from this model could also influence the discussion regarding future control programmes, with the added benefit of allowing it to be tailored to specific areas where data are available and reliable. There are many questions regarding control of fasciolosis that arose from developing this model and more accurate records of prevalence would be invaluable. What effect could vaccination have on future predictions of the disease? Should efforts be focused in developing an effective vaccine or on controlling the intermediate host? Could genetically modified hosts that are resistant or less susceptible to *F. hepatica* be an option? There is still a lot to learn about this disease and its control and the use of more accurate and abundant data can make this model an excellent tool to shape new control programmes to the specific needs of specific areas. The potential for adaptation of this model to changes in climate and farm practices make it even more useful if we consider no other model can predict what effect these parameters will have in future infections.

Bibliography

K A Abbott, M Taylor, and L A Stubbings. Sustainable control of parasites in sheep sustainable worm control strategies 4th Edition. (June), 2012.

M Abrous, D Rondelaud, and G Dreyfuss. The stress of Lymnaea truncatula just before miracidial exposure with *Fasciola hepatica* increased the prevalence of infection. *Experimental Parasitology*, 99(1):49–51, 2001.

M L Adrien, A L Schild, C Marcolongo-Pereira, F Letícia, J L Ruas, F B Grecco, and M B Raffi. Acute fasciolosis in cattle in southern Brazil. *Pesquisa Veterinária*, 33:705–709, 2013.

W M S Al-Habbib. *The effect of constant and changing temperatures on the development of the larval stages of* Fasciola hepatica. PhD thesis, University of Dublin, 1974.

W M S Al-Habbib and J N R Grainger. The effect of constant and changing temperature on the rate of development of the eggs and the larval stages of *Fasciola hepatica*. *Proceedings of the Royal Irish Academy. Section B: Biological, Geological, and Chemical Science*, 83B:281–290, 1983.

M A Álvarez Sánchez, R C Mainar-Jaime, J Pérez-García, and F A Rojo-Vázquez. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. *Veterinary Record*, 159:424–425, 2006.

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

S J Andrews. *Fasciolosis*. Cambridge: University Press, 1999.

K Ashrafi, M. D Bargues, S O'Neill, and S Mas-Coma. Fascioliasis: A worldwide parasitic disease of importance in travel medicine. *Travel Medicine and Infectious Disease*, 12: 636–649, 2014.

J C Boray. Experimental fascioliasis in Australia. *Advances in parasitology*, 7:95–210, 1969.

J C Boray and K Enigk. Laboratory studies on the survival and infectivity of *Fasciola hepatica* and *F. gigantica* metacercariae. *Z Tropenmed Parasitol*, 15:324–331, 1964.

C C Boswell and A Smith. The use of a fluorescent pigment to record the distribution by cattle of traces of faeces from dung pats. *Grass and Forage Science*, 31:135–136, 1976.

R V Brunsdon. Liver fluke *Fasciola hepatica* in sheep and cattle in New Zealand and its control. *New Zealand Veterinary Journal*, 15(1-2):9–23, 1967.

R P Bryan and J D Kerr. The grazing behavior of cattle in relation to the sampling of infective nematode larvae on pasture. *Veterinary Parasitology*, 30:73–82, 1988.

C Caminade, J van Dijk, M Baylis, and D Williams. Modelling recent and future climatic suitability for fasciolosis in europe. *Geospatial Health*, 9:301–308, 2015. URL `http://www.geospatialhealth.net/index.php/gh/article/view/352`.

J Charlier, K Soenen, E De Roeck, W Hantson, E Ducheyne, F Van Coillie, R De Wulf, G Hendrickx, and J Vercruysse. Longitudinal study on the temporal and micro-spatial

distribution of *Galba truncatula* in four farms in Belgium as a base for small-scale risk mapping of *Fasciola hepatica*. *Parasites & Vectors*, 7:528, 2014a.

J Charlier, J Vercruysse, E Morgan, J van Dijk, and D J L Williams. Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. *Parasitology*, 141:326–335, 2014b.

N O Christensen, P Nansen, and F Frandsen. Molluscs interfering with capacity of *Fasciola hepatica* miracidia to infect Lymnaea truncatula. *Parasitology*, 73:161–167, 1976.

J Claridge, P Diggle, C M McCann, G Mulcahy, R Flynn, J McNair, S Strain, M Welsh, M Baylis, and D J L Williams. *Fasciola hepatica* is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nature communications*, 3:853, 2012.

J R Claxton, J Sutherst, P Ortiz, and M J Clarkson. The effect of cyclic temperatures on the growth of *Fasciola hepatica* and *Lymnaea viatrix*. *Veterinary Journal*, 157(2): 166–171, 1999.

G C Coles, C Bauer, F H Borgsteede, S Geerts, T R Klei, M Taylor, and P J Waller. Methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 44:35–44, 1992.

J Connolly. Prediction of the growth of organisms under conditions varying with time. *Proceedings of the Royal Irish Academy Section B-Biological Geological and Chemical Science*, 79:271–280, 1979.

A. Cywińska. Epidemiology of fascioliasis in human endemic areas. *Journal of Helminthology*, 79(2005):207–216, 2016. doi: 10.1079/JOH2005296.

J De Brie. Traict de l'estat, science et pratique de l'art de bergerie. *Letter addressed to Charles V of France*, 1379.

C P de Souza, K G Magalhaes, L K J Passos, G C P dos Santos, F Ribeiro, and N Katz. Aspects of the maintenance of the life cycle of *Fasciola hepatica* in *Lymnaea columella* in Minas Gerais, Brazil. *Memorias Do Instituto Oswaldo Cruz*, 97:407–410, 2002.

K Dittmar and W R Teegen. The presence of *Fasciola hepatica* (liver-fluke) in humans and cattle from a 4,500 year old archaeological site in the Saale-Unstrut valley, Germany. *Memórias do Instituto Oswaldo Cruz*, 98 Suppl 1:141–3, 2003.

Edgar Howard Dixon, K Eand Mercer. The formation of the cyst wall of the metacercaria of *Fasciola hepatica* l. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 77: 345–360, 1967.

K E Dixon. The relative suitability of sheep and cattle as hosts for the liver fluke, *Fasciola hepatica*. *Journal of Helminthology*, 38:203–212, 1964.

K. E. Dixon. The physiology of excystment of the metacercaria of *Fasciola hepatica* L. *Parasitology*, 56:431–456, 1966.

G Dreyfuss and D Rondelaud. *Fasciola hepatica* - A study of the shedding of cercariae from *Lymnaea truncatula* raised under constant conditions of temperature and photoperiod. *Parasite-Journal De La Societe Francaise De Parasitologie*, 1:401–404, 1994.

G Dreyfuss, D Rondelaud, and C Vareille-Morel. Oviposition of *Lymnaea truncatula* infected by *Fasciola hepatica* under experimental conditions. *Parasitology research*, 85: 589–593, 1999.

G Dreyfuss, N Alarion, P Vignoles, and D Rondelaud. A retrospective study on the metacercarial production of *Fasciola hepatica* from experimentally infected *Galba truncatula* in central France. *Parasitology Research*, 98:162–166, 2006.

Els Ducheyne, Johannes Charlier, Jozef Vercruysse, Laura Rinaldi, Annibale Biggeri, Janina Demeler, Christina Brandt, Theo De Waal, Nikolaos Selemetas, Johan Höglund, Jaroslaw Kaba, Slawomir J Kowalczyk, and Guy Hendrickx. Modelling the spatial distribution of *Fasciola hepatica* in dairy cattle in Europe. *Geospatial health*, 9:261–270, 2015.

I Fairweather. Liver fluke isolates: a question of provenance. *Veterinary Parasitology*, 176:1–8, 2011.

C B Field, V R Barros, D J Dokken, K J Mach, M D Mastrandrea, T E Bilir, M Chatterjee, K L Ebi, Y O Estrada, R C Genova, B Girma, E S Kissel, A N Levy, S MacCracken, P R Mastrandrea, and White L L. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. *Cambridge University Press*, pages 11–32, 2014.

R J Flynn, C Mannion, O Golden, O Hacariz, and G Mulcahy. Experimental *Fasciola hepatica* infection alters responses to tests used for diagnosis of bovine tuberculosis. *Infection and Immunity*, 75(3):1373–81, 2007.

N J Fox, P C L White, C J McClean, G Marion, A Evans, and M R Hutchings. Predicting impacts of climate change on *Fasciola hepatica* risk. *Plos One*, 6:1–9, 2011.

C P H Gaasenbeek, H J Over, N Noorman, and W A de Leeuw. An epidemiological study of *Fasciola hepatica* in the Netherlands. *Veterinary Quarterly*, 14:140–144, 1992.

G Gettinby and W Byrom. Weather based computer experiments on parasites. *Preventative Veterinary Medicine*, 11:293–308, 1991.

E A Goodall, S G McIlroy, R M McCracken, E M McLoughlin, and S M Taylor. A mathematical forecasting model for the annual prevalence of Fasciolosis. *Agricultural Systems*, 36:231–240, 1991.

T K Graczyk and B Fried. Development of *Fasciola hepatica* in the intermediate host. *In Dalton J.P. (Ed.)*, pages 31–46, 1999.

A Gutiérrez, G Perera, M Yong, J Sánchez, and W Lin. Life-history traits of *Fossaria cubensis* (Gastropoda: *Lymnaeidae*) under experimental exposure to *Fasciola hepatica* (Trematoda: Digenea). *Memórias do Instituto Oswaldo Cruz*, 95:747–52, 2000.

R E B Hanna, C McMahon, S Ellison, H W Edgar, P E Kajugu, A Gordon, D Irwin, J P Barley, F E Malone, G P Brennan, and I Fairweather. *Fasciola hepatica*: A comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in northern ireland using faecal egg counting, coproantigen {ELISA} testing and fluke histology. *Veterinary Parasitology*, 207:34–43, 2015.

F A Happich and J C Boray. Quantitative diagnosis of chronic Fasciolosis .2. Estimation of daily total egg production of *Fasciola hepatica* and number of adult flukes in sheep by faecal egg counts. *Australian Veterinary Journal*, 45:329–331, 1969a.

F A Happich and J C Boray. Quantitative diagnosis of chronic Fasciolosis .1. Comparative studies on quantitative faecal examinations for chronic *Fasciola hepatica* infection in sheep. *Australian Veterinary Journal*, 45:332–337, 1969b.

C D Hawkins and R S Morris. Depression of productivity in sheep infected with *Fasciola hepatica*. *Veterinary Parasitology*, 4:341–351, 1978.

P B Heppleston. Life history and population fluctuations of *Lymnaea truncatula* (Mull), snail vector of Fascioliasis. *Journal of Applied Ecology*, 9:235–248, 1972.

J K M Hodasi. The effects of *Fasciola hepatica* on *Lymnaea truncatula*. *Parasitology*, 65: 359–369, 1972.

M J Hope Cawdery, K L Strickland, A Conway, and P J Crowe. Production effects of liver fluke in cattle I. The effects of infection on live weight gain, feed intake and food conversion efficiency in beef cattle. *British Veterinary Journal*, 133(2):145–159, 1977.

A H A Hussein, I M Hassan, and R M A Khalifa. Development and hatching mechanism of Fasciola eggs, light and scanning electron microscopic studies. *Saudi Journal of Biological Sciences*, 17(3):247–251, 2010.

R M Kaplan. *Fasciola hepatica*: A review of the economic impact in cattle and considerations for control. *Veterinary Therapeutics*, 2(1):40–50, 2001.

S B Kendall. Bionics of *Lymnaea truncatula* and the parthenitae of *Fasciola hepatica* under drought conditions. *Journal of Helminthology*, 23:57–68, 1949.

S B Kendall. The Life-History of *Limnaea truncatula* under laboratory conditions. *Journal of Helminthology*, 27(1-2):17–28, 1953.

S B Kendall. Relationships between the species of *Fasciola* and their molluscan hosts. *Advances in parasitology*, 3:59–98, 1965.

S B Kendall and F S McCullough. The emergence of the cercariae of *Fasciola hepatica* from the snail *Limnaea truncatula*. *Journal of Helminthology*, 25:77–92, 1951.

S B Kendall and C B Ollerenshaw. Effect on nutrition on growth of *Fasciola hepatica* in its snail host. *Proceedings of the Nutrition Society*, 22:41–46, 1963.

K Khallayoune, B E Stromberg, A Dakkak, and J B Malone. Seasonal dynamics of *Fasciola hepatica* burdens in grazing timahdit sheep in Morocco. *International Journal of Parasitology*, 21(3):307–314, 1991.

R A Knight. Experimental cross infections of *Fasciola hepatica* in lambs and calves. *The Journal of Parasitology*, 64:601–605, 1978.

W H Krull. New snail and rabbit hosts for *Fasciola hepatica* Linn. *The Journal of Parasitology*, 20(1):49–52, 1933.

W H Krull. Notes on the hatchability and infectivity of refrigerated eggs of *Fasciola hepatica* Linn. *Proceedings of the Iowa Academy of Science*, 41:309–311, 1934.

W H Krull. The number of cercariae of *Fasciola hepatica* developing in snails infected with a single miracidium. *The Helminthological Society of Washington*, 8(2):55–58, 1941.

C G Lee, S K Kim, and C Y Lee. Rapid growth of *Lymnaea viridis*, the intermediate host of *Fasciola hepatica*, under laboratory conditions. *Veterinary Parasitology*, 51:327–331, 1994.

C G Lee, S H Cho, and C Y Lee. Metacercarial production of *Lymnaea viridis* experimentally infected with *Fasciola hepatica*. *Veterinary Parasitology*, 58(4):313–318, 1995.

M Luzón-Peña, M Gormez-Bautista, and F A Rojo-Vazquez. The contribution of *Fasciola hepatica* eggs deposited during the autumn to the snail infection in a central area of spain. *Research and Reviews in Parasitology*, 51:75–79, 1991.

M Luzón-Peña, F A Rojo-Vazquez, and M Gomez-Bautista. The overwintering of *Fasciola hepatica* eggs under semi-arid and temperate Mediterranean climate (Madrid, Spain). *Journal of Veterinary Medicine. Series B*, 39(5):369–375, 1992.

M Luzón-Peña, F A Rojo-Vazquez, and M Gómez-Bautista. The overwintering of eggs, intramolluscal stages and metacercariae of *Fasciola hepatica* under the temperatures of a Mediterranean area (Madrid, Spain). *Veterinary Parasitology*, 55:143–148, 1994.

M Luzón-Peña, F Rojo-Vázquez, and M Gómez-Bautista. Seasonal availability of *Fasciola hepatica* metacercariae in a temperate Mediterranean area (Madrid, Spain). *Journal of Veterinary Medicine. Series B*, 42:577–585, 1995.

N MacKintosh and P Brophy. Measuring Triclabendazole resistance as part of a whole farm strategy for the control of liver fluke in sheep and cattle. Technical report, Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth, 2012.

C Mage, H Bourgne, J M Toullieu, D Rondelaud, and G Dreyfuss. *Fasciola hepatica* and *Paramphistomum daubneyi*: changes in prevalences of natural infections in cattle and in *Lymnaea truncatula* from central France over the past 12 years. *Veterinary Research*, 33(5):439–447, 2002.

J B Malone, R Gommes, J Hansen, J M Yilma, J Slingenberg, F Snijders, F Nachtergaele, and E Ataman. A geographic information system on the potential distribution and abundance of *Fasciola hepatica* and *F gigantica* in east Africa based on Food and Agriculture Organization databases. *Veterinary Parasitology*, 78:87–101, 1998.

María Martínez-Valladares and Francisco Antonio Rojo-Vázquez. Loop-mediated isothermal amplification (LAMP) assay for the diagnosis of fasciolosis in sheep and its application under field conditions. *Parasites & Vectors*, 9:73, 2016.

S Mas-Coma and M D Bargues. Human liver flukes: A review. *Research and Reviews in Parasitology*, 57:145–218, 1997.

S Mazeri, N Sargison, R F Kelly, B M de C Bronsvoort, and I Handel. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. *PloS One*, pages 1–22, 2016.

C M McCann, M Baylis, and D J L Williams. The development of linear regression models using environmental variables to explain the spatial distribution of *Fasciola hepatica* infection in dairy herds in England and Wales. *International Journal for Parasitology*, 40(9):1021–1028, 2010.

S G McIlroy, E A Goodall, D A Stewart, S M Taylor, and R M McCracken. A computerised system for the accurate forecasting of the annual prevalence of fasciolosis. *Preventive Veterinary Medicine*, 9:27–35, 1990.

C. McMahon, H.W.J. Edgar, R.E.B. Hanna, S.E. Ellison, A.M. Flanagan, M. McCoy, P.-E. Kajugu, A.W. Gordon, D. Irwin, J.E. Barley, F.E. Malone, G.P. Brennan, and I. Fairweather. Liver fluke control on sheep farms in northern ireland: A survey of changing

management practices in relation to disease prevalence and perceived triclabendazole resistance. *Veterinary Parasitology*, 216:72 – 83, 2016.

D McManus and J Dalton. Vaccines against the zoonotic trematodes schistosoma japonicum, *Fasciola hepatica* and *Fasciola gigantica*. *Parasitology*, 133:43–61, 2006.

A J McMichael, R E Woodruff, and S Hales. Climate change and human health: Present and future risks. *Lancet*, 367:859–869, 2006.

L Moll, C P Gaasenbeek, P Vellema, and F H Borgsteede. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. *Veterinary Parasitology*, 91:153–8, 2000.

E Morgan, J Charlier, G Hendrickx, A Biggeri, D Catalan, G von Samson-Himmelstjerna, J Demeler, E Müller, J van Dijk, F Kenyon, P Skuce, J Höglund, P O'Kiely, B van Ranst, T de Waal, L Rinaldi, G Cringoli, H Hertzberg, P Torgerson, A Wolstenholme, and J Vercruysse. Global Change and Helminth Infections in Grazing Ruminants in Europe: Impacts, Trends and Sustainable Solutions. *Agriculture*, 3:484–502, 2013.

NADIS.

P Nansen, F Frandsen, and N O Christensen. Study on snail location by *Fasciola hepatica* using radioisotopically labeled miracidia. *Parasitology*, 72(APR):163–171, 1976.

N G Nice. *Aspects of the biology of* Fasciola hepatica *and its intermediate host* Lymnaea truncatula. PhD thesis, University of York, 1979.

N G Nice and R A Wilson. A study of the effect of temperature on the growth of *Fasciola hepatica* in *Lymnaea truncatula*. *Parasitology*, 68:47–56, 1974.

L J O'Connor, L P Kahn, and S W Walkden-Brown. The effects of amount, timing and distribution of simulated rainfall on the development of Haemonchus contortus to the infective larval stage. *Veterinary parasitology*, 146:90–101, 2007.

C B Ollerenshaw. The approach to forecasting the incidence of fascioliasis over England and Wales 1958 to 1962. *Agricultural Meteorology*, 3(12):35–53, 1966.

C B Ollerenshaw. Some observations on epidemiology of Fascioliasis in relation to timing of molluscicide applications in control of disease. *Veterinary Record*, 88:152–164, 1971.

C B Ollerenshaw and W T Rowlands. A method of forecasting the incidence of fascioliasis in Angelsey. *Veterinary Record*, 71:591–598, 1959.

O W Olsen. Longevity of metacercariae of *Fasciola hepatica* on pastures in the upper coastal regions of Texas and its relationship to liver fluke control. *Journal of Parasitology*, 33:36–42, 1947.

H J Over. Ecological basis of parasite control: trematodes with special reference to fascioliasis. *Veterinary Parasitology*, 11(1):85–97, 1982.

E M Pantelouris. Environmental influences on the life-cycle of the liver-fluke, *Fasciola hepatica* L. *The Irish Naturalists' Journal*, 14(5):94–97, 1963.

H L Penman. Natural evaporation from open water, bare soil and grass. *Proceedings of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, 193 (1032):120–145, 1948.

S K Prah and C James. The influence of physical factors on the survival and infectivity of miracidia of *Schistosoma mansoni* and *S. haematobium* I. Effect of temperature and ultra-violet light. *Journal of Helminthology*, 51:73–85, 1977.

R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, 2013. URL `http://www.R-project.org/`.

C Rapsch, G Schweizer, F Grimm, L Kohler, C Bauer, P Deplazes, U Braun, and P R Torgerson. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. *International Journal for Parasitology*, 36(10-11):1153–8, 2006.

V Relf, B Good, E McCarthy, and T de Waal. Evidence of *Fasciola hepatica* infection in *Radix peregra* and a mollusc of the family Succineidae in Ireland. *Veterinary Parasitology*, 163(12):152–155, 2009.

V Relf, B Good, J P Hanrahan, E McCarthy, A B Forbes, and T DeWaal. Temporal studies on *Fasciola hepatica* in *Galba truncatula* in the west of Ireland. *Veterinary Parasitology*, 175(34):287–292, 2011.

E W Roberts. Studies on the life-cycle of Fasciola-hepatica (Linnaeus) and of its snail host, *Limnaea-(Galba)-truncatula* (Muller), in the field and under controlled conditions in the laboratory. *Annals of Tropical Medicine and Parasitology*, 44:187–206, 1950.

J A Roberts and Suhardono. Approaches to the control of fasciolosis in ruminants. *International Journal for Parasitology*, 26(8-9):971–981, 1996.

M C Rognlie, K L Dimke, S E Knapp, M C Rognlie, K L Dimke, and S E Knapp. Detection of *Fasciola hepatica* in infected intermediate hosts using RT-PCR. *The Journal of Parasitology*, 80(5):748–755, 1994.

A Rohatgi. Webplotdigitizer, 2015. URL `http://arohatgi.info/WebPlotDigitizer`.

D Rondelaud and G Dreyfuss. Variability of *Fasciola* infections in Lymnaea truncatula as a function of snail generation and snail activity. *Journal of Helminthology*, 71(2): 161–166, 1997.

D Rondelaud, C Denève, M Belfaiza, A Mekroud, M Abrous, M Moncef, and G Dreyfuss. Variability in the prevalence of infection and cercarial production in *Galba truncatula* raised on a high-quality diet. *Parasitology Research*, 92(3):242–245, 2004.

D Rondelaud, P Hourdin, P Vignoles, G Dreyfuss, and Jacques Cabaret. The detection of snail host habitats in liver fluke infected farms by use of plant indicators. *Veterinary Parasitology*, 181(24):166–173, 2011.

J G Ross. The Stormont "wet day" forecasting system for fascioliasis. *British Veterinary Journal*, 8:401–408, 1970.

S A Rowcliffe and C B Ollerenshaw. Observations on the bionomics of the egg of *Fasciola hepatica*. *Annals of Tropical Medicine and Parasitology*, 54:172–181, 1960.

K Sasithorn. Taxonomy and biology of liver flukes. *Acta Tropica*, 88:177–186, 2003.

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

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

S Shaka and P Nansen. Epidemiology of Fascioliasis in Denmark - Studies on the sea-

sonal availability of metacercariae and the parasite stages overwintering on pasture. *Veterinary Parasitology*, 5:145–154, 1979.

J N Shaw and B T Simms. Studies in fascioliasis in Oregon sheep and goats. *Agricultural Experiment Station Oregon State Agricultural College*, 1930.

C D Shubkin, M W White, M S Abrahamsen, C Matthew, S E Knapp, C D Shubkin, M W White, M S Abrahamsen, M C Rognlie, and S E Knapp. A nucleic acid-based test for detection of *Fasciola hepatica*. *The Journal of Parasitology*, 78(5):817–821, 1992.

G Smith. Models of macroparasitic infections in domestic ruminants: a conceptual review and critique. *Rev Sci Tech*, 30:447–456, 2011.

T W Spithill, P M Smooker, J L Sexton, E Bozas, C A Morrison, J Creaney, and J C Parsons. *Development of vaccines against* Fasciola hepatica. CABI Publishing, 1999.

B E Stromberg. Environmental factors influencing transmission. *Veterinary Parasitology*, 72:247–64, 1997.

A P Thomas. The natural history of the liver fluke and the prevention of rot. *J. Royal Agric. Soc. England*, 1883.

H Toet, D M. Piedrafita, and T W. Spithill. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. *International Journal for Parasitology*, 44:915–927, 2014.

P R Torgerson and J R Claxton. Epidemiology and Control. *In Fasciolosis Dalton*, pages 113–149, 1999.

J Turner, A Howell, C McCann, C Caminade, R G. Bowers, D Williams, and M Baylis. A model to assess the efficacy of vaccines for control of liver fluke infection. *Scientific Reports*, 6(September 20 s15):23345, 2016.

M A Valero and S Mas-Coma. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitologica*, 47:17–22, 2000.

M A Valero, M Panova, A M Comes, R Fons, and S Mas-Coma. Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. *Journal of Parasitology*, 88(2):308–313, 2002.

J van Dijk and E R Morgan. The influence of temperature on the development, hatching and survival of *Nematodirus battus* larvae. *Parasitology*, 135:269–283, 2008.

J van Dijk, G P David, G Baird, and E R Morgan. Back to the future: Developing hypotheses on the effects of climate change on ovine parasitic gastroenteritis from historical data. *Veterinary Parasitology*, 158:73–84, 2008.

J van Dijk, N D Sargison, F Kenyon, and P J Skuce. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. *Animal*, 4(3):377–392, 2010.

I N Vasileva. A study of ontogenesis in the trematode *Fasciola hepatica* in the Moscow region (in Russian). *Zoologicheskii Zhurnal*, 39:1478–1484, 1960.

P Vignoles, N Alarion, V Bellet, G Dreyfuss, and D Rondelaud. A 6- to 8-day periodicity in cercarial shedding occurred in some *Galba truncatula* experimentally infected with *Fasciola hepatica*. *Parasitology Research*, 98:385–388, 2006.

P. Vignoles, D. Rondelaud, and G. Dreyfuss. Characteristics of *Fasciola hepatica* infections in *Galba truncatula* originating from riverbank populations. *Journal of Helminthology*, 85:28–32, 2011.

S M Walker, E M Hoey, H L Fletcher, G Brennan, I Fairweather, and A Trudgett. Stage-specific differences in fecundity over the life-cycle of two characterized isolates of the liver fluke, *Fasciola hepatica*. *Parasitology*, 133:209–216, 2006.

RT Watson, MC Zinyowera, and Rh Moss. The Regional Impacts of Climate Change: An Assessment of Vulnerability. *Intergovernmental Panel on Climate Change*, pages 1–27, 1997.

P. H. Williams and R. J. Haynes. Effect of sheep, deer and cattle dung on herbage production and soil nutrient content. *Grass and Forage Science*, 50:263–271, 1995.

R A Wilson and J Denison. The Parasitic Castration and Gigantism of *Lymnaea truncatula* Infected with the Larval Stages of Fasciola-Hepatica. *Zeitschrift Fur Parasitenkunde-Parasitology Research*, 61:109–119, 1980.

R A Wilson and S L Taylor. Effect of variations in host and parasite density on level of parasitization of *lymnaea truncatula* by Fasciola hepatica. *Parasitology*, 76:91–98, 1978.

R A Wilson, R Pullin, and J Denison. An investigation of the mechanism of infection by digenetic trematodes: the penetration of the miracidium of Fasciola hepatica into its snail host *Lymnaea truncatula* °C. *Parasitology*, 63:491–506, 1971.