Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant *Fasciola hepatica*

J. HODGKINSON[1]*, K. CWIKLINSKI[1], N. J. BEESLEY[1], S. PATERSON[2] *and* D. J. L. WILLIAMS[1]

[1] *Veterinary Parasitology, Institute of Infection and Global Health, University of Liverpool, Liverpool, L3 5RF, UK*
[2] *Centre for Genomic Research, University of Liverpool, Liverpool L69 7ZB, UK*

(*Received 11 February 2013; revised 2 April 2013; accepted 2 April 2013; first published online 31 May 2013*)

SUMMARY

Despite years of investigation into triclabendazole (TCBZ) resistance in *Fasciola hepatica*, the genetic mechanisms responsible remain unknown. Extensive analysis of multiple triclabendazole-susceptible and -resistant isolates using a combination of experimental *in vivo* and *in vitro* approaches has been carried out, yet few, if any, genes have been demonstrated experimentally to be associated with resistance phenotypes in the field. In this review we summarize the current understanding of TCBZ resistance from the approaches employed to date. We report the current genomic and genetic resources for *F. hepatica* that are available to facilitate novel functional genomics and genetic experiments for this parasite in the future. Finally, we describe our own non-biased approach to mapping the major genetic loci involved in conferring TCBZ resistance in *F. hepatica*.

Key words: *Fasciola hepatica*, *Galba truncatula*, SNPs, genome, microsatellite, population genetics.

INTRODUCTION

The liver fluke, *Fasciola hepatica* is an economically important trematode parasite pathogen of livestock which frequently impacts on the health and welfare of cattle and sheep worldwide. Evidence from various sources suggests that the prevalence of infection has increased considerably in recent years for a variety of reasons – changing climate, changing farming practices and increased animal movements (van Dijk *et al*. 2010). It is suggested that climate change is at least partly responsible for the increase in the prevalence of *F. hepatica* in the UK; moreover, warmer winters and wetter summers, which are predicted to occur in the UK over the next 50 years, combined with growing concerns about drug resistance (Charlier *et al*. 2012) are likely to exacerbate the financial and welfare impact of fasciolosis on livestock production (Fox *et al*. 2011). We also know that flukes modulate the host immune system and affect diagnosis and susceptibility to other pathogens including bovine tuberculosis (Claridge *et al*. 2012).

The life cycle of *F. hepatica* includes many pre-parasitic stages which develop in the environment or the snail intermediate host, *Galba truncatula*, where they undergo a clonal expansion (Fig. 1). The infective stage is the metacercaria which is found encysted on herbage and following ingestion by grazing herbivores develops to patency in 10–14 weeks. Temperature and rainfall are the principle determinants affecting the life cycle and hence the prevalence and intensity of *F. hepatica* infection (McCann *et al*. 2010).

F. hepatica is regarded by the WHO as a re-emerging zoonosis and of major concern in many developing countries. It has been estimated that 17 million people are at risk of infection and new hyper-endemic regions have been identified in Vietnam, Egypt and Iran, in addition to the well established hyper-endemic region in the altiplano of the South American Andes. The drug of choice to treat human fasciolosis, triclabendazole (TCBZ), is identical to one used to control fasciolosis in livestock. The emergence of anthelmintic resistance, in particular to TCBZ, by *F. hepatica* populations is an urgent concern to UK agriculture and a threat to human and animal health and welfare in endemic areas (González *et al*. 2011)

The majority of anthelmintic resistance studies focus on nematode species, where association of mutations in target genes are made with a resistance phenotype. The assumption that resistance is conferred primarily by single nucleotide polymorphisms (SNPs) or deletions in the coding regions of target genes has advanced our understanding for some parasite:drug interactions, such as benzimidazole resistance in *Haemonchus contortus*, but is proving

* Corresponding author. Veterinary Parasitology, Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool, L3 5RF, UK. Tel: +44 (0)151 7950223. E-mail: jhodgkin@liverpool.ac.uk

 © Cambridge University Press 2013
doi:10.1017/S0031182013000528

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

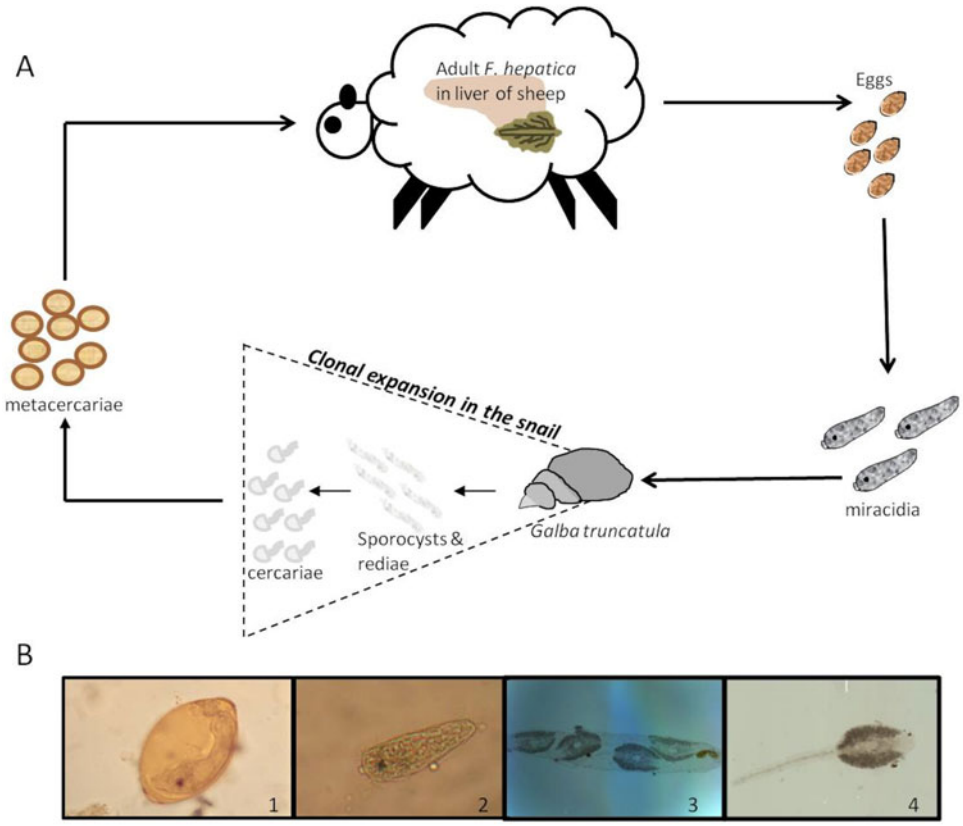

Fig.1. (A) Schematic showing the life cycle of *F. hepatica*. Eggs are produced by adults in the bile ducts of the definitive host (e.g. sheep) and are passed in the faeces. A key aspect of the life cycle that lends it to genetic manipulation is the ability of a single miracidium to hatch from an egg, infect the snail intermediate host and give rise to several hundred clonal cercariae that are subsequently released from the snail. These metacercariae encyst on pasture and are ingested by the definitive host; (B) Selected images from experimental infections: 1. Embryonated egg containing miracidium, 2. Free miracidum, 3. Redia from an infected snail, 4. Cercaria released from an infected snail.

to be a less effective approach for other parasite:drug combinations (Gilleard, 2006). The potential for anthelmintic resistance to be complex and multigenic in nature, differing between parasite species and/or isolates of the same species, and involving yet unidentified genes as potential candidates, identifies the need for alternative approaches.

In this review, we discuss a novel genetic mapping approach to identify the major genetic loci involved in conferring TCBZ resistance in *F. hepatica*. In order to appreciate this approach it is first necessary to review the current knowledge of TCBZ resistance mechanisms in *F. hepatica* based on either candidate gene approaches or descriptive studies of parasites exposed to TCBZ *in vivo* or *in vitro*. Given that the approach we have taken relies on genome-wide markers to map resistance-conferring genes it is important to consider the availability of genomic and genetic resources for this parasite.

TCBZ RESISTANCE

Since its introduction in the 1980s the benzimidazole, TCBZ, has been adopted worldwide as the drug of choice for controlling *F. hepatica* infection in livestock and humans. Whilst other drugs can be used to target parasitic stages later in the fluke life cycle TCBZ remains the only drug available for the control of acute fasciolosis due to its efficacy against flukes less than four weeks old. However, this heavy reliance on TCBZ to treat sheep and cattle in particular, has resulted in resistance to TCBZ, which was first reported in fluke populations in Australia in 1995 and is now well established in the Netherlands, Ireland, Spain and all UK regions (Fairweather, 2005; Brennan *et al*. 2007). In the UK, our own studies in England and Wales showed that TCBZ resistance was evident on seven out of 25 farms analysed (Daniel *et al*. 2012). The movement of livestock around the country facilitates the spread of TCBZ resistant (TCBZ-R) *F. hepatica* populations throughout the UK with the potential for considerable economic consequences to livestock production such as that recently reported by Sargison and others (Sargison *et al*. 2010; Sargison and Scott, 2011). In humans, TCBZ is the only drug used to treat fascioliasis, the zoonotic disease caused by infection with *F. hepatica,* and the first report of the failure of TCBZ to treat human *F. hepatica* infection has now emerged (Winkelhagen *et al*. 2012).

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

Detecting TCBZ resistance

The issue of guidelines for the detection of anthelmintic resistance in nematodes has been promoted by the World Association for the Advancement of Veterinary Parasitology (WAAVP) and remains an active discussion; however, in the case of *F. hepatica* no gold standard exists for defining an isolate based on its drug sensitivity *in vivo* or *in vitro*. A comprehensive review of methods for detecting resistance falls outside the scope of this article but key considerations in this area involve the ongoing evaluation of methods applicable to the live host such as the faecal egg count reduction tests (FECRT) and coproantigen reduction test (Flanagan *et al*. 2011*a*, *b*; Daniel *et al*. 2012). Whilst these approaches are open to interpretation, multiple studies demonstrate TCBZ efficacy *in vivo* using treatment and necropsy approaches. These studies involve either a natural host, commonly sheep (for example see Coles *et al*. 2000, 2001; McConville *et al*. 2009; Gordon *et al*. 2012) or the rat 'model host' system (for example see Meaney *et al*. 2007). In most cases the *in vivo* efficacy of drugs is established using a procedure of experimental infection ($\sim$200 and $\sim$20 metacercariae for sheep and rats, respectively) followed by TCBZ treatment, typically at 12 weeks post-infection, to establish efficacy against adult stages. Necropsy is performed typically at 14 days post-treatment followed by recovery of surviving adult fluke for enumeration and descriptive studies. Variations to these protocols exist when drug efficacy against different stages of the parasite and/or TCBZ metabolites are under evaluation (Halferty *et al*. 2008, 2009). An important consideration of the *in vitro* or *in vivo* descriptive studies reporting structural or morphological changes in TCBZ-R *vs* triclabendazole-susceptible (TCBZ-S) *F. hepatica* is the question of the long-term effect of these alterations over the course of infection. Even though steps were taken recently to demonstrate the validity of these changes in an *in vivo* system (Devine *et al*. 2012), given that the flukes were still alive post-treatment these may merely reflect transient changes in fluke architecture in response to the drug from which the flukes may well recover. More recently, an *in vitro* tool for the diagnosis of TCBZ resistance was reported (Fairweather *et al*. 2012), but this is proving difficult to take beyond a proof of concept due to the toxicity of DMSO required to dissolve TCBZ at the higher concentrations (Hartley, personal communication).

Mechanisms of TCBZ resistance

Understanding the basis of TCBZ resistance has been hampered because, despite extensive investigation, the mode of action of TCBZ remains unresolved. Extensive investigations into the mechanisms of TCBZ resistance in *F. hepatica* have been the focus of other reviews (Fairweather, 2005, 2009, 2011) and are only outlined here.

Several $\alpha-$ and β-tubulin isotypes are expressed in adult flukes but, in contrast to the situation with ruminant nematodes and benzimidazole drugs, there is a lack of evidence to support the binding of TCBZ to tubulin molecules or a role for tubulin mutations in TCBZ resistance (Ryan *et al*. 2008). There is more convincing evidence of a role for the altered uptake, efflux and metabolism of TCBZ (Alvarez *et al*. 2005). It has been shown *in vitro* that metabolism of TCBZ to the active metabolites TCBZ.SO and TCBZ.SO_2 occurs to a greater extent in TCBZ-R than TCBZ-S flukes (Robinson *et al*. 2004; Alvarez *et al*. 2005). A role for drug metabolism was further supported by detailed electron microscopic analysis of adult flukes incubated *in vitro* with combinations of TCBZ and TBCZ.SO and inhibitors of the cytochrome P450 (Devine *et al*. 2010*a*, 2011) and flavin monooxygenase metabolic pathways (Devine, 2010*b*). More recently, this has been demonstrated in an *in vivo* model system (Devine *et al*. 2012). Drug efflux mechanisms have been highlighted as one of several potential contributory factors to TCBZ resistance (Alvarez *et al*. 2005; Mottier *et al*. 2006) and are a current focus of anthelmintic resistance studies in other helminths (Prichard and Roulet, 2007; James and Davey, 2009). Possible transporters for drug efflux include the P-glycoproteins (Pgps), of which a homologue has been identified in *F. hepatica*, although this form is only expressed in immature flukes (Reed *et al*. 1998). A recent study reported single nucleotide polymorphisms (SNPs) in the nucleotide-binding domain of Pgp genes that differed between TCBZ-S and TCBZ-R adult *F. hepatica* from the field (Wilkinson *et al*. 2012). Given the small number of flukes examined in the study ($n=3$ TCBZ-R and $n=1$ TCBZ-S), the significance of these SNPs remains to be determined. Overall these biochemical results suggest that any combination of several potential pathways may be responsible for TCBZ resistance (Brennan *et al*. 2007).

GENOMIC, TRANSCRIPTOMIC AND PROTEOMIC RESOURCES FOR *F. HEPATICA*

The limitations of candidate gene-association studies and the value of employing genetic and genomic approaches to anthelmintic resistance have already been highlighted (Gilleard, 2006). This latter approach, combined with an understanding of the population genetics of a parasite offers an attractive complementary approach to understanding how parasites develop resistance. The added advantage is that not only are genes identified in a non-biased way but their relative importance to the resistant phenotype can also be assessed. In the case of the ruminant nematode *H. contortus*, these studies are already yielding interesting insights into the biology of this

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

Table 1. Currently available nucleotide sequence for *F. hepatica*

Originating Institute	Stage and source of *F. hepatica*	Type of sequence data	Web reference	Sequence reference and total sequence available	Date made available
University of Liverpool	1 adult (sheep at abattoir, UK)	Transcriptome	http://www.ebi.ac.uk/ena/data/view/ERP000012	UoL1: ~119 MB. ~ 38 000 ESTs	2008
Wellcome Trust Sanger Institute	Adults(sheep and cattle, Iran/Spain)	ESTs	ftp://ftp.sanger.ac.uk/pub/pathogens/Fasciola/	Fhn3, Fh4, Fhn5; 11000 ESTs	2007
University of Melbourne	3 adult s (Victoria, Australia)	Transcriptome	http://bioinfosecond.vet.unimelb.edu.au/index.html	590 927 reads, 61 105 contigs and 74540 singletons	2010
University of Queensland	1 adult (Geelong Strain, Australia)	Mitochondrial genome	AF216697	14462 bp	2001
Universidad de la República, Uruguay	1200 NEJ	ESTs	dbEST: GT740211 to GT741887	1684 ESTs	2010
University of Liverpool	1 adult (Shrewsbury isolate)	Genome	NA	NK – ongoing project	NYA
International Food-Borne Trematodiases Community	NK	Genome	http://www.genome.gov/Pages/Research/DER/PathogensandVectors/FBT_white_paper_14Dec2010_final.pdf	NK – ongoing project	NYA

NEJ, Newly excysted juveniles; EST,expressed sequence tags; bp, base pairs; NA, Not Available; NK, Not Known, NYA, Not Yet Available.

parasite and drug resistance mechanisms in particular (Redman *et al*. 2012). In order for this approach to be relevant to *F. hepatica* it is important to review the genomic and genetic tools available and identify the need for further development of these resources.

TCBZ has been reported to induce a broad range of effects, with several potential modes of action (Brennan *et al*. 2007; Chemale *et al*. 2010). Investigating the nature of resistance to this anthelmintic therefore requires a broad analysis of these helminths, using all available genomic, transcriptomic and proteomic datasets. To date, several studies have been carried out using proteomic tools to investigate the secretome of *F. hepatica* (Jefferies *et al*. 2001; Morphew *et al*. 2007; Robinson *et al*. 2009), including the response to TCBZ (Chemale *et al*. 2010) and 'virulence'-associated proteins (reviewed by McVeigh *et al*. 2012). One study integrated transcriptomic and proteomic data to analyse the potential differences in expression during migration of *F. hepatica* parasites in the liver (Robinson *et al*. 2009), but it was limited by the lack of available mRNA and EST sequences from Wellcome Trust Sanger Institute (WTSI; Table 1). In-depth sequencing of the *F. hepatica* and *F. gigantica* transcriptomes using next-generation sequencing technologies has only recently been carried out (Young *et al*. 2010, 2011; Paterson *et al*. unpublished data; Table 1). Young and colleagues interrogated the transcriptomic data using Gene Ontology (GO) and metabolic pathways to identify key biological processes and pathways, and used data from closely related helminths to investigate potential *Fasciola* species-specific genes (Young *et al*. 2010, 2011). In-depth analysis of the *Fasciola* species transcriptome has been limited to date to specific gene families; for example, the SCP/TAPS proteins (Cantacessi *et al*. 2012), the glutathione transferase superfamily (Morphew *et al*. 2012) and cathepsins (Morphew *et al*. 2011). As these analyses develop they are likely to reveal interesting insights into parasite biology, including resistance mechanisms, and will provide platforms for the discovery of novel diagnostics, vaccines and drugs to support intervention strategies in the future.

Recently there has been increased interest in microRNAs (miRNAs) and their involvement in anthelmintic resistance (Devaney *et al*. 2010). One study investigating miRNAs in *F. hepatica* and *F. gigantica* identified 16 and 19 miRNAs, respectively in the two helminth datasets (Xu *et al*. 2012). Only a small number of ESTs (WTSI) were used for analysis of potential targets of the miRNAs and the genome of the related helminth, *Schistosoma japonicum*, was used as a reference - both of which are likely to have limitations.

Such studies have highlighted the fact that to elucidate the nature of complex gene families, such as the cathepsins (McVeigh *et al*. 2012), to identify

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

species-specific sequences of interest and to map genes associated with TCBZ resistance, a *F. hepatica* genomic resource is essential. To date, no publically available *Fasciola* species genome sequence datasets are available, although several groups, including ourselves, are currently sequencing the *F. hepatica* and *F. gigantica* genomes (Table 1).

GENETIC RESOURCES FOR *F. HEPATICA*

Population genetic analyses are a vital component of anthelmintic resistance studies, facilitating our understanding of the origin, evolution and, most importantly, spread of resistance genes in populations (Gilleard and Beech, 2007). An understanding of the geographical variation of *F. hepatica* will also allow interpretation of candidate gene-association studies. Given that *Fasciola* species undergo parthenogenesis and can both self- and cross-fertilize, they are an interesting and complex group of parasites in which to study population genetic structure and gene flow. This is compounded by the fact that there is a clonal expansion in the snail intermediate host and infection can occur in multiple mammalian definitive hosts, which may impact on the levels of gene flow, aggregation of transmission and parasitic load. All these factors affect the effective population size and the degree of inbreeding which in turn impacts on the spread of resistance genes.

Ribosomal and mitochondrial DNA markers

A number of randomly amplified genetic markers or protein electrophoresis methods has been developed and employed over the last 15 years to facilitate characterization and differentiation of *F. hepatica* and/or *F. gigantica* isolates (Li and Quiros 2001; Vilas *et al*. 2002; Vargas *et al*. 2003; Ramadan and Saber, 2004; Aldemir, 2006; McGarry *et al*. 2007; Alasaad *et al*. 2008). The ability of these markers to determine population sub-structuring and/or genetic diversity remains either untested or is limited (Ellsworth *et al*. 1993; Backeljau *et al*. 1995; Beveridge, 1998; Vázquez-Prieto *et al*. 2011). Ribosomal and mitochondrial DNA of *Fasciola* species offer a more promising region to exploit for studying heterogeneity.

Amplification of the entire 28S rDNA (~4 kb) gene revealed few polymorphisms within *F. hepatica* and *F. gigantica* isolates from different geographical locations (Marcilla *et al*. unpublished data cited in Marcilla *et al*. 2002) however, a 618 bp region was subsequently developed as a PCR-restriction fragment length polymorphism assay (PCR-RFLP) (Marcilla *et al*. 2002). Similarly the complete mitochondrial genome of *F. hepatica* (~14·5 kb) was sequenced from two geographically distinct isolates (Australia and Salt Lake City, Table 1) but showed limited (<1%) intra-specific variation (Le *et al*. 2001). More recently a PCR-RFLP of the ribosomal ITS-1 sequence was used to determine heterogeneity of *Fasciola* species in Asian countries (Ichikawa and Itagaki, 2010). Other ribosomal regions, such as the ITS-2 rDNA, have been sequenced and compared amongst isolates of *F. hepatica* (Erensoy *et al*. 2009); used to investigate the origin of triploidy in *Fasciola* species (Itagaki and Tsutsumi, 1998) and to differentiate between *F. hepatica, F. gigantica* and intermediate forms of *Fasciola* species from sheep and cattle (Itagaki and Tsutsumi, 1998; Ali *et al*. 2008). Detection of SNPs in fragments of a 28S rDNA gene region identified two basic lineages of *F. hepatica* and showed a distinct difference between north-eastern and south-eastern European isolates (Walker *et al*. 2007; Teofanova *et al*. 2011). However, no explicit lineages or genetic structuring were identified in these isolates based on the β-tubulin 3 gene (Teofanova *et al*. 2011). Analysis of SNPs in NAD1 and COX1 genes from twenty locations across eastern Europe and western Asia revealed little genetic structuring between the two populations (eastern Europe and western Asia) possibly indicating high gene flow between them due to the migration of the definitive host (Semyenova *et al*. 2006).

Despite its widespread popularity as a genetic marker there are limitations to using mitochondrial DNA such is its potential to undergo genetic recombination and positive selection, thus reducing its value as a neutral marker of population diversity (Galtier *et al*. 2009).

Microsatellite markers

In order to study the spread of anthelmintic resistance, biparentally inherited markers such as microsatellites offer a more comprehensive approach (Johnson *et al*. 2006). To date, few microsatellite markers have been identified for *F. hepatica* and have been limited in their application (Hurtrez-Boussès *et al*. 2004; Dar *et al*. 2011). The most extensive study to date used a combination of microsatellites ($n=4$) and allozymes ($n=8$) to analyse populations of *F. hepatica* ($n=587$) from sheep and cattle in north west Spain (Vilas *et al*. 2012). Existing studies have concentrated on the adult stage of the *F. hepatica* life cycle, often with only small sample sizes and have identified a need for greater analysis and development of genome-wide microsatellites, to unravel the population genetic structure of *F. hepatica*. From our genomic data we have developed a reliable new panel of ~15 markers (Cwiklinski *et al*. unpublished data) to genetically profile adult parasites which we are using to determine the current population genetic structure of *F. hepatica* in the UK. In addition, a sub-panel of microsatellite markers (8 polymorphic loci; Cwiklinski *et al*. unpublished data) has been optimized to genotype batches of 50 metacercariae,

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

Table 2. A summary of the provenance of *F. hepatica* isolates available for TCBZ resistance studies

Isolate name	Date of isolation	Resistance Status	Parasite stage evaluated	Ploidy	References[a]
Cullompton	1998	TCBZ-S	Adult	Aspermic, triploid Undergoes parthenogenesis	Walker *et al*. (2004); McCoy *et al*. (2005)
Fairhurst	1985	TCBZ-S	Adult	Diploid	Boray (1990); Walker *et al*. (2004); McCoy *et al*. (2005); Flanagan *et al*. (2011*a*)
Oberon	1999	TCBZ-R	Adult	Diploid	Walker *et al*. (2004)
Sligo	1998	TCBZ-R	3 day, 4 week, 12 week pi fluke	Phenotype 1: fully developed spermatids Phenotype 2: arrested at spermatid stage	Coles *et al*. (2000); Coles and Stafford, (2001); McCoy *et al*. (2005); McConville *et al*. (2009)
Leon	2006	TCBZ-S, CLOR-R (+IVM)	Adult	Diploid	Alvarez-Sanchez *et al*. (2006); Flanagan *et al*. (2011*a*)
Shrewsbury	2006	TCBZ-S	Adult	Diploid	Ridgeway Research, personal communication: TCBZ-S: 97% efficacy reported in sheep ($n=9$)
South Gloucester	2008	TCBZ-S	Adult	ND	Ridgeway Research, personal communication
Sunny corner	1989	TCBZ-S	Adult 2 week, 4 week, 6 week pi fluke	ND	(Fairweather (2009), citing Boray, personal communication
Dutch	2001	TCBZ-R	Adult	ND	Gaasenbeek *et al*. (2001)

[a] References are cited for studies reporting resistance status, if no published studies available then personal communications cited. TCBZ-S, triclabendazole susceptible; TCBZ-R, triclabendazole resistant; CLOR-R(+IVM) , clorsulon resistant (given as combination with ivermectin); Diploid, $2n=20$; Triploid, $3n=30$; week, week; pi, post infection; ND, Not determined.

miracidia and eggs. This approach offers significant promise for population genetic and molecular epidemiological studies of *F. hepatica* in animals and humans in the future, avoiding the need to passage cercariae through a host to produce adults for analysis as has been described for *Schistosoma haematobium* (Dabo *et al*. 1997) and *Opisthorcis viverrini* (Jex *et al*. 2012; Laoprom *et al*. 2012).

Application of population genetic markers to TCBZ resistance in F. hepatica

The application of genetic markers to explore the development and spread of resistance genes in *F. hepatica* is limited to just a few studies on a small number of parasites. A 510 bp fragment of the 28S rDNA gene was sequenced for adult *F. hepatica*, from sheep in northwest Spain, which were either fully susceptible to albendazole (ALB), clorsulon (CLOR) and TCBZ ($n=10$); ALB/CLOR resistant ($n=5$); ALB/TCBZ resistant ($n=5$); or ALB/CLOR/TCBZ resistant ($n=5$). SNPs were detected but their significance in resistance remains unknown (Vara-Del Río *et al*. 2007). An alternative RFLP approach using three loci of the mitochondrial genome was used to profile genetically small numbers of adult *F. hepatica* from sheep and cattle from several geographical locations and from two laboratory isolates; Fairhurst (TCBZ-S, $n=18$ fluke) and Oberon (TCBZ-R, $n=18$ fluke) (Walker *et al*. 2007). The RFLP study was extended to *F. hepatica* ($n=422$) from cattle ($n=29$) displaying either a TCBZ-S or -R phenotype (Walker *et al*. 2011). Whilst it is tempting to speculate about how these studies impact on our understanding of the mechanisms and spread of TCBZ resistance (Walker *et al*. 2011; Vilas *et al*. 2012) more comprehensive analyses are required to further our understanding of how resistant genes emerge and once established, how they flow both within and amongst different mammalian hosts from different geographical locations.

F. HEPATICA ISOLATES AND ANTHELMINTIC RESISTANCE STUDIES

A number of *in vivo* and *in vitro* studies has identified biological differences in isolates and genetic analysis showing that there is considerable heterogeneity of fluke populations in the field, both of which are important considerations when interpreting experimental studies. The wider liver fluke research community relies on a small number of providers of *F. hepatica* metacercariae (for example, Baldwin Aquatics, Oregon, US) and, as identified in a recent review, few isolates of *F. hepatica* are available for drug efficacy studies (Fairweather, 2011). A summary of the isolates currently available for resistance studies can be found in Table 2. Historically, studies on

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

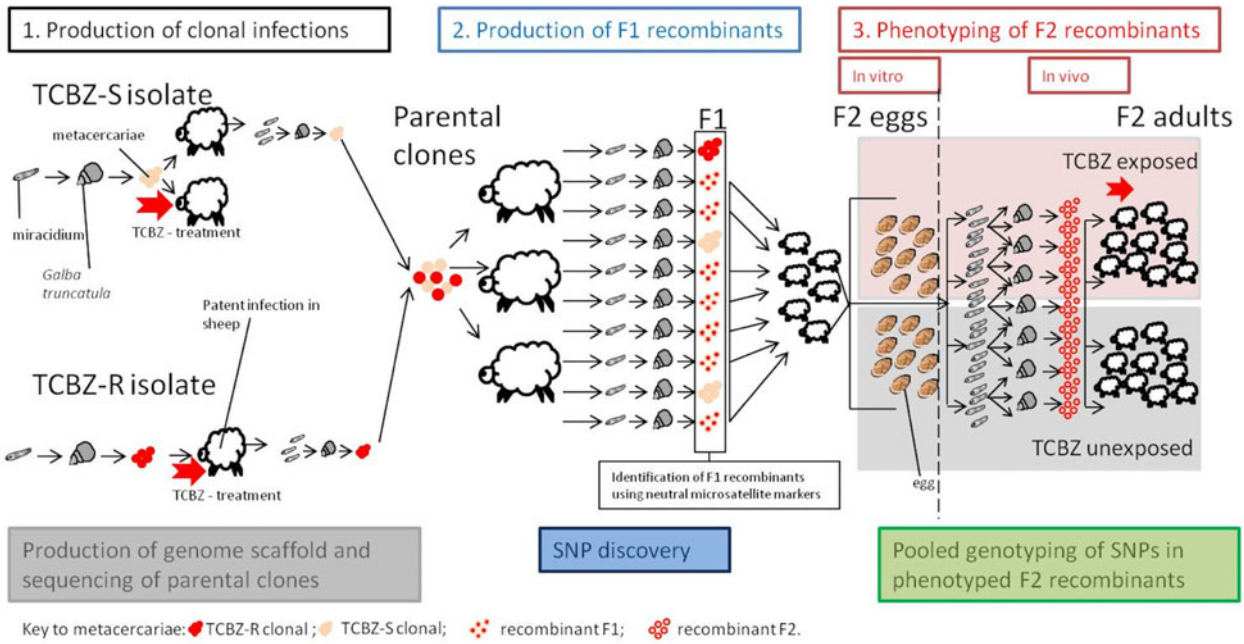

Fig. 2. Schematic representation of the experimental approach used to map TCBZ resistance in *F. hepatica*. The experiment is divided into three phases: (1) the production of clonal infections; (2) the production of F1 recombinants and (3) the production and phenotyping of F2 recombinants. Concurrent production of a genomic scaffold of *F. hepatica* supports discovery of SNPs in parental clones to allow association of SNPs in the pooled genotyping of phenotyped F2 recombinants either exposed or unexposed to TCBZ. The identification of F1 recombinants is performed using genome-derived microsatellites as neutral markers of parental clones. Metacercariae from self-fertilization or parthenogenesis, which display parental markers only, are shown for completeness but are not used for subsequent production of F2 recombinants. TCBZ, triclabendazole; TCBZ-S, triclabendazole susceptible; TCBZ-R, triclabendazole resistant.

TCBZ resistance in *F. hepatica* have relied on four laboratory isolates, the TCBZ–S Fairhurst and Cullompton isolates and the TCBZ-R, Sligo and Oberon isolates (Table 2). All of these isolates have been maintained in the laboratory for more than a decade and the serial passage required for their maintenance, in particular the clonal expansion in the snail, plus the self fertilization of adult parasites, is likely to render the isolate more homogenous with time and potentially less relevant to the field. To ensure the laboratory isolate is representative of the original population requires active steps to derive metacercariae from multiple miracidial:snail infections using eggs recovered from multiple hosts. Little reference is made to the production of metacercariae in any study and studies of population genetics on laboratory isolates are scarce. The observation of a single haplotype in 18 adult fluke of the Fairhurst isolate is consistent with increased homogeneity over time (Walker *et al*. 2007) as is the lack of heterogeneity observed in the Shrewsbury fluke isolates (Cwiklinski, unpublished data). Whilst *in vivo* infection and drug-treatment studies serve to confirm an isolate's phenotype, the question of their relevance in a field context still remains. The maintenance of the widely used TCBZ-S Cullompton isolate has resulted in an isolate which is aspermic, where there is no meiosis and which is also triploid (Fletcher *et al*. 2004); a situation which contrasts with diploid flukes found in naturally infected hosts (Fletcher *et al*. 2004; Reblánová *et al*. 2011; Beesley, unpublished data). The fact that a fluke isolate cannot be archived at any point necessitates serial passage but it is important that due consideration of this process is given by academic and commercial suppliers of metacercariae.

MAPPING TCBZ RESISTANCE GENES IN *F. HEPATICA*

Currently underway in our laboratory is a project which employs genome-wide SNPs to map TCBZ resistance genes in genetically manipulated *F. hepatica* (see Fig. 2). The advantage of this approach is that no assumption is made about the mechanisms of TCBZ resistance and our findings will be directly relevant in the current UK field situation. The approach controls for the complex reproductive biology and demography of *F. hepatica* that would complicate a study of field isolates alone. A draft genome sequence for *F. hepatica* has been produced in order to support SNP discovery and the application of genome-wide SNPs to map regions of the genome associated with resistance is underway. In order to dissect the spread of genes associated with TCBZ resistance we are taking an experimental approach by crossing TCBZ-S and TCBZ-R clones of *F. hepatica*

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

and mapping TCBZ-R genes through subsequent F1 and F2 populations (Fig. 2).

A draft *F. hepatica* genome has been generated by a combination of Roche 454 and Illumina sequence data and assembled into contigs and scaffolds. Annotation is underway, using automated pipelines to generate gene models based on the *F. hepatica* transcriptome and schistosome protein sequence. Even in this rough form, this draft genome will greatly improve the sequence resources available to the research community (Table 1), although generating improved functional annotation will likely be a community effort requiring significant investment.

To ensure use of isolates representative of the current UK field situation and to avoid issues associated with laboratory isolates we have identified TCBZ-R field isolates from south Wales and north-west England, UK where high levels of TCBZ resistance have been reported (Daniel *et al*. 2012). Following confirmation of their TCBZ resistance status in experimental infections in sheep (in collaboration with Ridgeway Research), we have exploited the life-cycle of *F. hepatica* to generate clonal lines of TCBZ-R and TCBZ-S isolates. Metacercariae from single TCBZ-resistant miracidial:snail infections have been used to infect sheep concurrently infected with a clone of the TCBZ-S Shrewsbury isolate (Table 2). Key to the success of this approach is the ability to distinguish F1 and F2 progeny that have undergone genetic recombination. These experiments are complicated by the fact that flukes can both self- and cross-fertilize, although there is evidence that cross fertilization predominates (Hanna *et al*. 2008). A sub-panel of 8 microsatellites (Cwiklinski, unpublished data) has been used to profile parental clones genetically and is being employed as neutral markers to track the genotypes of the recombinant progeny and allow subsequent identification of F1 and F2 recombinants. A snail infected with one miracidium can produce up to 600 cercariae, all genetic clones of the infecting miracidium, allowing a proportion of cercariae to be genotyped to identify F1 recombinants but leaving sufficient metacercariae to infect a host for the purposes of producing F2.

Ultimately, the project relies on comparing the frequency of SNP alleles derived from the resistant parental clone and linked to the TCBZ resistance locus (or loci) in TCBZ exposed parasites (only resistant parasites) relative to untreated controls (a mixture of resistant and susceptible parasites). Unlinked SNPs (neutral loci) will show no difference in frequency between TCBZ and control parasites. Pooled genotyping on a number of replicates followed by analysis of allele frequencies to associate SNPs (and hence genomic regions) to TCBZ-resistance will be performed on F2 populations. Once SNPs are identified they will be mapped to the draft *F. hepatica* genome sequence to identify contigs and scaffolds (and ultimately genes) associated with TCBZ resistance.

FUTURE PERSPECTIVES

The identification of genomic regions associated with TCBZ resistance will enable future work to investigate the biochemical function of candidate resistance genes and to perform finer-scale disequilibrium mapping of resistance in field samples. The genome-wide scan for SNPs linked to TCBZ resistance will highlight many genomic scaffolds and may determinate whether these scaffolds are physically linked, i.e. whether there is a single TCBZ resistance locus or several loci, and independent validation for the association between genomic regions and TCBZ resistance. Whilst an initial analysis of the predicted coding sequences lying within the contigs can be performed to determine whether they contain any obvious candidates for TCBZ resistance, the more detailed molecular analysis of candidate resistance loci will form the basis of future work. This newly available genomic resource, together with the recent developments in functional genomics tools such as RNAi and luciferase reporter gene activity assays (McGonigle *et al*. 2008; Rinaldi *et al*. 2008) will promote studies at the molecular level to further facilitate our understanding of these important parasites.

ACKNOWLEDGEMENTS

The authors would like to thank Dr James LaCourse for his contribution to the writing of the BBSRC project to map TCBZ resistance, Miss Katherine Allen for her work maintaining the snail colonies and fluke isolates at the University of Liverpool and Mrs Catherine Hartley for her work with the egg hatch assay and parasite recovery. We would like to thank Ms Paula Martin, Mr Oliver Gladstone and staff at Ridgeway Research for their support and advice with experimental infection and parasite maintenance. We would like to thank Roger Daniel and Rowan Wood at the AHVLA for their support in the identification of TCBZ-R field isolates.

FINANCIAL SUPPORT

The work described here was generously supported by the BBSRC grant number BB/I002480/1. Ms Nicola Beesley is a PhD student funded by the Institute of Infection and Global Health, University of Liverpool.

REFERENCES

Alasaad, S., Li, Q. Y., Lin, Q., Martín-Atance, P., Granados, J. E., Díez-Baños, P., Pérez, J. M. and Zhu, X. Q. (2008). Genetic variability among *Fasciola hepatica* samples from different host species and geographical localities in Spain revealed by the novel SRAP marker. *Parasitology Research* **103**, 181–186. doi: 10.1007/s00436-008-0952-1.

Aldemir, O. S. (2006). Differentiation of cattle and sheep originated *Fasciola hepatica* by RAPD-PCR. *Revue de Médecine Vétérinaire* **157**, 65–67.

Ali, H., Ai, L., Song, H. Q., Ali, S., Lin, R. Q., Seyni, B., Issa, G. and Zhu, X. Q. (2008). Genetic characterisation of *Fasciola* samples from different host species and geographical localities revealed the existence of *F. hepatica* and *F. gigantica* in Niger. *Parasitology Research* **102**, 1021–1024. doi: 10.1007/s00436-007-0870-7.

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

Alvarez, L. L., Solana, H. D., Mottier, M. L., Virkel, G. L., Fairweather, I. and Lanusse, C. E. (2005). Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology* **131**, 501–510. doi: 10.1017/S0031182005007997.

Alvarez-Sanchez, M. A., Mainar-Jaime, R. C., Perez-Garcia, J. and Rojo-Vazquez, F. A. (2006). Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. *Veterinary Record* **159**, 424–425. doi: 10.1136/vr.159.13.424.

Backeljau, T., De Bruyn, L., De Wolf, H., Jordaens, K., Van Dongen, S., Verhagen, R. and Winnepenninckx, B. (1995). Random amplified polymorphic DNA (RAPD) and parsimony methods. *Cladistics* **11**, 119–130. doi: 10.1111/j.1096-0031.1995.tb00083.x.

Beveridge, I. (1998). Allozyme electrophoresis – difficulties encountered in studies on helminths. *International Journal for Parasitology* **28**, 973–979. doi: 10.1016/S0020-7519(98)00047-2.

Boray, J. C. (1990). Drug resistance in *Fasciola hepatica*. In *Resistance of Parasites to Antiparasitic Drugs* (ed. Boray, J. C., Martin, P. J. and Roush, R. T.), pp. 51–60. MSD AGVET, Rahway, NJ.

Brennan, G. P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G. and Brophy, P. M. (2007). Understanding triclabendazole resistance. *Experimental and Molecular Pathology* **82**, 104–109. doi: 10.1016/jyexmp.2007.01.009X.

Cantacessi, C., Hofmann, A., Young, N. D., Broder, U., Hall, R. S., Loukas, A. and Gasser, R. B. (2012). Insights into SCP/TAPS proteins of liver flukes based on large-scale bioinformatic analyses of sequence datasets. *PLoS ONE* **7**, e31164. doi: 10.1371/journal.pone.0031164.

Charlier, J., Hostens, M., Jacobs, J., Van Ranst, B., Duchateau, L. and Vercruysse, J. (2012). Integrating fasciolosis control in the dry cow management: the effect of closantel treatment on milk production. *PLoS ONE* **7**, 8. doi: 10.1371/journal.pone.0043216.

Chemale, G., Perally, S., LaCourse, E. J., Prescott, M. C., Jones, L. M., Ward, D., Meaney, M., Hoey, E., Brennan, G. P., Fairweather, I., Trudgett, A. and Brophy, P. M. (2010). Comparative proteomic analysis of triclabendazole response in the liver fluke *Fasciola hepatica*. *Journal of Proteome Research* **9**, 4940–4951. doi: 10.1021/pr1000785.

Claridge, J., Diggle, P., McCann, C. M., Mulcahy, G., Flynn, R., McNair, J., Strain, S., Welsh, M., Baylis, M. and Williams, D. J. (2012). *Fasciola hepatica* is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nature Communications* **3**, 853. doi: 10.1038/ncomms1840.

Coles, G. C., Rhodes, A. C. and Stafford, K. A. (2000). Activity of closantel against triclabendazole-resistant *Fasciola hepatica*. *Veterinary Record* **146**, 504. doi: 10.1136/vr.146.17.504-a.

Coles, G. C. and Stafford, K. A. (2001). Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole-resistant *Fasciola hepatica*. *Veterinary Record* **148**, 723–724. doi: 10.1136/vr.148.23.723.

Dabo, A., Durand, P., Morand, S., Diakite, M., Langand, J., Imbert-Establet, D., Doumbo, O. and Jourdane, J. (1997). Distribution and genetic diversity of *Schistosoma haematobium* within its bulinid intermediate hosts in Mali. *Acta Tropica* **66**, 15–26. doi: 10.1016/S0001-706X(97)00670-0.

Daniel, R., van Dijk, J., Jenkins, T., Akca, A., Mearns, R. and Williams, D. J. L. (2012). A composite faecal egg count reduction test to detect resistance to tricalbendazole in *Fasciola hepatica*. *Veterinary Record* **171**, 153. doi: 10.1136/vr.100588.

Dar, Y., Amer, S., Courtioux, B. and Dreyfuss, G. (2011). Microsatellite analysis of *Fasciola* spp. in Egypt. *Parasitology Research* **109**, 1741–1744. doi: 10.1007/s00436-011-2520-3.

Devaney, E., Winter, A. D. and Britton, C. (2010). microRNAs: a role in drug resistance in parasitic nematodes? *Trends in Parasitology* **26**, 428–433. doi: 10.1016/j.pt.2010.05.003.

Devine, C., Brennan, G. P., Lanusse, C. E., Alvarez, L. I., Trudgett, A., Hoey, E. and Fairweather, I. (2010*a*). Enhancement of the drug susceptibility of a triclabendazole-resistant isolate of *Fasciola hepatica* using the metabolic inhibitor ketoconazole. *Parasitology Research* **107**, 337–353. doi: 10.1007/s00436-010-1866-2.

Devine, C., Brennan, G. P., Lanusse, C. E., Alvarez, L. I., Trudgett, A., Hoey, E. and Fairweather, I. (2010*b*). Potentiation of triclabendazole sulphoxide-induced tegumental disruption by methimazole in a triclabendazole-resistant isolate of *Fasciola hepatica*. *Parasitology Research* **106**, 1351–1363. doi: 10.1007/s00436-010-1806-1.

Devine, C., Brennan, G. P., Lanusse, C. E., Alvarez, L. I., Trudgett, A., Hoey, E. and Fairweather, I. (2011). Erratum to: inhibition of triclabendazole metabolism *in vitro* by ketoconazole increases disruption to the tegument of a triclabendazole-resistant isolate of *Fasciola hepatica*. *Parasitology Research* **109**, 1209–1223. doi: 10.1007/s00436-011-2554-6.

Devine, C., Brennan, G. P., Lanusse, C. E., Alvarez, L. I., Trudgett, A., Hoey, E. and Fairweather, I. (2012). Potentiation of triclabendazole action *in vivo* against a triclabendazole-resistant isolate of *Fasciola hepatica* following its co-administration with the metabolic inhibitor, ketoconazole. *Veterinary Parasitology* **184**, 37–47. doi: 10.1016/j.vetpar.2011.08.006.

Ellsworth, D. L., Rittenhouse, K. D. and Honeycutt, R. L. (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14**, 214–217.

Erensoy, A., Kuk, S. and Ozden, M. (2009). Genetic identification of *Fasciola hepatica* by ITS-2 sequence of nuclear ribosomal DNA in Turkey. *Parasitology Research* **105**, 407–412. doi: 10.1007/s00436-009-1399-8.

Fairweather, I. (2005). Triclabendazole: new skills to unravel an old(ish) enigma. *Journal of Helminthology* **79**, 227–234. doi: 10.1079/JOH2005298.

Fairweather, I. (2009). Triclabendazole progress report, 2005–2009: an advancement of learning? *Journal of Helminthology* **83**, 139–150. doi: 10.1017/S0022149X09321173.

Fairweather, I. (2011). Liver fluke isolates: a question of provenance. *Veterinary Parasitology* **176**, 1–8. doi: 10.1016/j.vetpar.2010.12.011.

Fairweather, I., McShane, D. D., Shaw, L., Ellison, S. E., O'Hagan, N. T., York, E. A., Trudgett, A. and Brennan, G. P. (2012). Development of an egg hatch assay for the diagnosis of triclabendazole resistance in *Fasciola hepatica*: Proof of concept. *Veterinary Parasitology* **183**, 249–259. doi: 10.1016/j.vetpar.2011.07.023.

Flanagan, A., Edgar, H. W. J., Forster, F., Gordon, A., Hanna, R. E. B., McCoy, M., Brennan, G. P. and Fairweather, I. (2011*b*). Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. *Veterinary Parasitology* **176**, 34–42. doi: 10.1016/j.vetpar.2010.10.037.

Flanagan, A., Edgar, H. W. J., Gordon, A., Hanna, R. E. B., Brennan, G. P. and Fairweather, I. (2011*a*). Comparison of two assays, a faecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. *Veterinary Parasitology* **176**, 170–176. doi: 10.1016/j.vetpar.2010.10.057.

Fletcher, H. L., Hoey, E. M., Orr, N., Trudgett, A., Fairweather, I. and Robinson, M. W. (2004). The occurrence and significance of triploidy in the liver fluke, *Fasciola hepatica*. *Parasitology* **128**, 69–72. doi: 10.1017/S003118200300427X.

Fox, N. J., White, P. C., McClean, C. J., Marion, G., Evans, A. and Hutchings, M. R. (2011). Predicting impacts of climate change on *Fasciola hepatica* risk. *PLoS ONE* **6**:e16126. doi: 10.1371/journal.pone.0016126.

Gaasenbeek, C. P. H., Moll, L., Cornelissen, J. B. W. J., Vellema, P. and Borgsteede, F. H. M. (2001). An experimental study on triclabendazole resistance of *Fasciola hepatica* in sheep. *Veterinary Parasitology* **95**, 37–43. doi: 10.1016/S0304-4017(00)00413-1.

Galtier, N., Nabholz, B., Glémin, S. and Hurst, G. D. D. (2009). Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology* **18**, 4541–4550. doi: 10.1111/j.1365-294X.2009.04380.x.

Gilleard, J. (2006). Understanding anthelmintic resistance: the need for genomics and genetics. *International Journal for Parasitology* **36**, 1227–1239. doi: 10.1016/j.ijpara.2006.06.010.

Gilleard, J. S. and Beech, R. N. (2007). Population genetics of anthelmintic resistance in parasitic nematodes. *Parasitology* **134**, 1133–1146. doi: 10.1017/S0031182007000066.

González, L. C., Esteban, J. G., Bargues, M. D., Valero, M. A., Ortiz, P., Náquira, C. and Mas-Coma, S. (2011). Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province. *Peru Acta Tropica* **120**, 119–129. doi: 10.1016/j.actatropica.2011.07.002.

Gordon, D., Zadoks, R. and Skuce, P. (2012). Confirmation of triclabendazole resistance in liver fluke in the UK. *Veterinary Record* **171**, 159–160. doi: 10.1136/vr.e5381.

Halferty, L., Brennan, G. P., Hanna, R. E., Edgar, H. W., Meaney, M. M., McConville, M., Trudgett, A., Hoey, L. and Fairweather, I. (2008). Tegumental surface changes in juvenile *Fasciola hepatica* in response to treatment *in vivo* with triclabendazole. *Veterinary Parasitology* **155**, 49–58. doi: 10.1016/j.vetpar.2008.04.011.

Halferty, L., Brennan, G. P., Trudgett, A., Hoey, L. and Fairweather, I. (2009). Relative activity of triclabendazole metabolites against the liver fluke, *Fasciola hepatica*. *Veterinary Parasitology* **159**, 126–138. doi: 10.1016/j,vetpar.2008.10.007.

Hanna, R. E. B., Edgar, H., Moffett, D., McConnell, S., Fairweather, I., Brennan, G. P., Trudgett, A., Hoey, E. M., Cromie, L., Taylor, S. M. and Daniel, R. (2008). *Fasciola hepatica*:

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

histology of the testis in egg producing adults of several laboratory-maintained isolates grown to maturity in cattle and sheep and in flukes from naturally infected hosts. *Veterinary Parasitology* **157**, 222–234. doi: 10.1016/j.vetpar.2008.07.022.

Hurtrez-Boussès, S., Durand, P., Jabbour-Zahab, R., Guégan, J. F., Meunier, C., Bargues, M. D., Mas-Coma, S. and Renaud, F. (2004). Isolation and characterization of microsatellite markers in the liver fluke (*Fasciola hepatica*). *Molecular Ecology Notes* **4**, 689–690. doi: 10.1111/j.1471-8286.2004.00786.x.

Ichikawa, M. and Itagaki, T. (2010). Discrimination of the ITS1 types of *Fasciola* spp. based on a PCR-RFLP method. *Parasitology Research* **106**, 757–761. doi: 10.1007/s00436-010-1724-2.

Itagaki, T. and Tsutsumi, K. (1998). Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. *International Journal for Parasitology* **28**, 777–781. doi: 10.1016/S0020-7519(98)00037-X.

James, C. E. and Davey, M. W. (2009). Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. *International Journal for Parasitology* **39**, 213–220. doi: 10.1016/j.ijpara.2008.06.009.

Jefferies, J. R., Campbell, A. M., van Rossum, A. J., Barrett, J. and Brophy, P. M. (2001). Proteomic analysis of *Fasciola hepatica* excretory-secretory products. *Proteomics* **1**, 1128–1132. doi: 10.1002/1615-9861(200109)1:9<1128::AID-PROT1128>3.0.CO;2-0.

Jex, A. R., Young, N. D., Sripa, J., Hall, R. S., Scheerlinck, J. P., Laha, T., Sripa, B. and Gasser, R. B. (2012). Molecular changes in *Opisthorchis viverrini* (Southeast Asian liver fluke) during the transition from the juvenile to the adult stage. *PLoS Neglected Tropical Diseases* **6**, e1916. doi: 10.1371/journal.pntd.0001916.

Johnson, P. C. D., Webster, L. M. I., Adam, A., Buckland, R., Dawson, D. A. and Keller, L. F. (2006). Abundant variation in micro-satellites of the parasitic nematode *Trichostrongylus tenuis* and linkage to a tandem repeat. *Molecular and Biochemical Parasitology* **148**, 210–218. doi: 10.1016/j.molbiopara.2006.04.011.

Laoprom, N., Sithithaworn, P., Andrews, R. H., Ando, K., Laha, T., Klinbunga, S., Webster, J. P., Petney, T. N. (2012). Population genetic structuring in *Opisthorchis viverrini* over various spatial scales in Thailand and Lao PDR. *PLOS Neglected Tropical Diseases* **6**, e1906. doi: 10.1371/journal.pntd.0001906.

Le, T. H., Blair, D. and McManus, D. P. (2001). Complete DNA sequence and gene organization of the mitochondrial genome of the liverfluke, *Fasciola hepatica* L. (Platyhelminthes; Trematoda). *Parasitology* **123**, 609–621. doi: 10.1017/S0031182001008733.

Li, G. and Quiros, C. F. (2001). Sequence-releated amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. *Theoretical and Applied Genetics* **103**, 455–461. doi: 10.1007/s001220100570.

Marcilla, A., Bargues, M. D. and Mas-Coma, S. (2002). A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Molecular and Cellular Probes* **16**, 327–333. doi: 10.1006/mcpr.2002.0429.

McCann, C. M., Baylis, M. and Williams, D. J. (2010). Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. *Veterinary Record* **166**, 612–617. doi: 10.1136/vr.b4836.

McConville, M., Brennan, G. P., Flanagan, A., Edgar, H. W. J., Hanna, R. E. B., McCoy, M., Gordon, A. W., Castillo, R., Hernandez-Campos, A. and Fairweather, I. (2009). An evaluation of the efficacy of compound alpha and triclabendazole against two isolates of *Fasciola hepatica*. *Veterinary Parasitology* **162**, 75–88. doi: 10.1016/j.vetpar.2009.02.004.

McCoy, M. A., Fairweather, I., Brennan, G. P., Kenny, J. M., Elison, S. and Forbes, A. B. (2005). The efficacy of nitroxynil and triclabendazole administered synchronously against juvenile triclabendazole-resistant *Fasciola hepatica* in sheep. *Research in Veterinary Science* **78**, 33. doi: 10.1016/j.rvsc.2005.02.001.

McGarry, J. W., Ortiz, P. L., Hodgkinson, J. E., Goreish, I. and Williams, D. J. L. (2007). PCR-based differentiation of *Fasciola* species (Trematoda: Fasciolidae), using primers based on RAPD-derived sequences. *Annals of Tropical Medicine and Parasitology* **101**, 415–421. doi: 10.1179/136485907X176508.

McGonigle, L., Mousley, A., Marks, N. J., Brennan, G. P., Dalton, J. P., Spithill, T. W., Day, T. A. and Maule, A. G. (2008). The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduces gut penetration. *International Journal for Parasitology* **38**, 149–155. doi: 10.1016/j.ijpara.2007.10.007.

McVeigh, P., Maule, A. G., Dalton, J. P. and Robinson, M. W. (2012). *Fasciola hepatica* virulence-associated cysteine peptidases: a systems biology perspective. *Microbes and Infection* **14**, 301–310. doi: 10.1016/j.micinf.2011.11.012.

Meaney, M., Allister, J., McKinstry, B., McLaughlin, K., Brennan, G. P., Forbes, A. B. and Fairweather, I. (2007). *Fasciola hepatica*: ultrastructural effects of a combination of triclabendazole and clorsulon against mature fluke. *Parasitology Research* **100**, 1091–1104. doi: 10.1007/s00436-006-0378-6.

Morphew, R. M., Eccleston, N., Wilkinson, T. J., McGarry, J., Perally, S., Prescott, M., Ward, D., Williams, D., Paterson, S., Raman, M., Ravikumar, G., Khalid Saifullah, M., Abbas Abidi, S. M., McVeigh, P., Maule, A. G., Brophy, P. M. and LaCourse, E. J. (2012). Proteomics and *in silico* approaches to extend understanding of the glutathione transferase superfamily of the tropical fluke *Fasciola gigantica*. *Journal of Proteome Research* **11**, 5876–5889. doi: 10.1021/pr300654w.

Morphew, R. M., Wright, H. A., LaCourse, E. J., Porter, J., Barrett, J., Woods, D. J. and Brophy, P. M. (2011). Towards delineating functions within the *Fasciola* secreted cathepsin I protease family by integrating *in vivo* based sub-proteomics and phylogenetics. *PLoS Neglected Tropical Diseases* **5**, e937. doi: 10.1371/journal.pntd.0000937.

Morphew, R. M., Wright, H. A., LaCourse, E. J., Woods, D. J. and Brophy, P. M. (2007). Comparative proteomics of excretory-secretory proteins released by the liver fluke *Fasciola hepatica* in sheep host bile and during *in vitro* culture ex host. *Molecular and Cellular Proteomics* **6**, 963–972. doi: 10.1074/mcp.M600375-MCP200.

Mottier, L., Alvarez, L., Fairweather, I. and Lanusse, C. (2006). Resistance-induced changes in triclabendazole transport in *Fasciola hepatica*: ivermectin reversal effect. *Journal of Parasitology* **92**, 1355–1360. doi: 10.1645/GE-922R.1.

Prichard, R. K. and Roulet, A. (2007). ABC transporters and beta-tubulin in macrocyclic lactone resistance: prospects for marker development. *Parasitology* **134**, 1123–1132. doi: 10.1017/S0031182007000091.

Ramadan, N. I. and Saber, L. M. (2004). Detection of genetic variability in nonhuman isolates of *Fasciola hepatica* and *Fasciola gigantica* by the RAPD-PCR technique. *Journal of the Egyptian Society of Parasitology* **34**, 679–689.

Reblánová, M., Špakulová, M., Orosová, M., Králová-Hromadová, I., Bazsalovicsová, E., Rajský, D. (2011). A comparative study of karyotypes and chromosomal location of rDNA genes in important liver flukes *Fasciola hepatica* and *Fascioloides magna* (Trematoda: Fasciolidae). *Parasitology Research* **109**, 1021–1028. doi: 10.1007/s00436-011-2339-y.

Redman, E., Sargison, N., Whitelaw, F., Jackson, F., Morrison, A., Bartley, D. J. and Gilleard, J. S. (2012). Introgression of ivermectin resistance genes into a susceptible *Haemonchus contortus* strain by multiple backcrossing. *PLoS Pathogens* **8**, 1–10. doi: e1002534.

Reed, M. B., Spithill, T. W., Strugnell, R. A. and Panaccio, M. (1998). *Fasciola hepatica*: stage-specific expression of novel gene sequences as identified by differential display. *Experimental Parasitology* **89**, 169–179.

Rinaldi, G., Morales, M. E., Cancela, M., Castillo, E., Brindley, P. J. and Tort, J. F. (2008). Development of functional genomic tools in trematodes: RNA interference and luciferase reporter gene activity in *Fasciola hepatica*. *PLoS Neglected Tropical Diseases* **9**, e260. doi:10.1371/journal.pntd.0000260.

Robinson, M. W., Lawson, J., Trudgett, A., Hoey, E. M. and Fairweather, I. (2004). The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. *Parasitology Research* **92**, 205–210. doi: 10.1007/s00436-003-1003-6.

Robinson, M. W., Menon, R., Donnelly, S. M., Dalton, J. P. and Ranganathan, S. (2009). An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*. *Molecular and Cellular Proteomics* **8**, 1891–1907. doi: 10.1074/mcp.M900045-MCP200.

Ryan, L. A., Hoey, E., Trudgett, A., Fairweather, I., Fuchs, M., Robinson, M. W., Chambers, E., Timson, D., Ryan, E., Fetwell, T., Ivens, A., Bentley, G. and Johnston, D. (2008). *Fasciola hepatica* expresses multiple α- and β-tubulin isotypes. *Molecular and Biochemical Parasitology* **159**, 73–78. doi: 10.1016/j.molbiopara.2008.02.001.

Sargison, N. D. and Scott, P. R. (2011). Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. *Veterinary Record* **168**, 159. doi: 10.1136/vr.c5332.

Sargison, N. D., Wilson, D. J., Penny, C. D. and Bartley, D. J. (2010). Unexpected production loss caused by helminth parasites in weaned beef calves. *Veterinary Record* **167**, 752–754. doi: 10.1136/vr.c5428.

Semyenova, S. K., Morozova, E. V., Chrisanfova, G. G., Gorokhov, V. V., Arkhipov, I. A., Moskvin, A. S., Movsessyan, S. O. and Ryskov, A. P. (2006). Genetic differentiation in Eastern European and Western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press

by mitochondrial NAD1 and COX1 genes. *Journal of Parasitology* **92**, 525–530. doi: 10.1645/GE-673R.1.

Teofanova, D., Kantzoura, V., Walker, S., Radoslavov, G., Hristov, P., Theodoropoulos, G., Bankov, I. and Trudgett, A. (2011). Genetic diversity of liver flukes (*Fasciola hepatica*) from Eastern Europe. *Infection, Genetics and Evolution* **11**, 109–115. doi: 10.1016/j.meegid.2010.10.002.

van Dijk, J., Sargison, N. D., Kenyon, F. and Skuce, P. J. (2010). Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. *Animal* **4**, 377–392. doi: 10.1017/S1751731109990991.

Vara-Del Río, M. P., Villa, H., Martinez-Valladares, M. and Rojo-Vázquez, F. A. (2007). Genetic heterogeneity of *Fasciola hepatica* isolates in the northwest of Spain. *Parasitology Research* **101**, 1003–1006. doi: 10.1007/s00436-007-0574-z.

Vargas, D., Vega, M. and González, C. G. (2003). Aproximación a una caracterización molecular de *Fasciola hepatica* por la técnica RAPDs – PCR. *Parasitología Latinoamericana* **58**, 11–16. doi: 10.4067/S0717-77122003000100002.

Vázquez-Prieto, S., Vilas, R., Mezo, M., González-Warleta, M., Ubeira, F. M. and Paniagua, E. (2011). Allozyme markers suitable for population genetic analysis of *Fasciola hepatica*. *Veterinary Parasitology* **176**, 84–88. doi: 10.1016/j.vetpar.2010.10.042.

Vilas, R., Paniagua, E., Outeiral, S. and Sanmartín, M. L. (2002). Electrophoretic and morphological differentiation of three sympatric species of the genus *Lecithochirium* (Trematoda: Hemiuridae), parasites of marine fishes. *Parasitology Research* **88**, 1055–1060. doi: 10.1007/s00436-002-0708-2.

Vilas, R., Vásquez-Prieto, S. and Paniagua, E. (2012). Contrasting patterns of population genetic structure of *Fasciola hepatica* from cattle and sheep: Implications for the evolution of anthelmintic resistance. *Infection, Genetics and Evolution* **12**, 45–52. doi: 10.1016/j.meegid.2011.10.010.

Walker, S. M., McKinstry, B., Boray, J. C., Brennan, G. P., Trudgett, A., Hoey, E. M., Fletcher, H. and Fairweather, I. (2004). Response of two isolates of *Fasciola hepatica* to treatment with triclabendazole *in vivo* and *in vitro*. *Parasitology Research* **94**, 427–438. doi: 10.1007/s00436-004-1222-5.

Walker, S. M., Johnston, C., Hoey, E. M., Fairweather, I., Borgsteede, F., Gaasenbeek, C., Prodöhl, P. A. and Trudgett, A. (2011). Population dynamics of the liver fluke, *Fasciola hepatica*: the effect of time and spatial separation on the genetic diversity of fluke populations in the Netherlands. *Parasitology* **138**, 215–223. doi: 10.1017/S0031182010001149.

Walker, S. M., Prodöhl, P. A., Fletcher, H. L., Hanna, R. E. B., Kantzoura, V., Hoey, E. M. and Trudgett, A. (2007). Evidence for multiple mitochondrial lineages of *Fasciola hepatica* (liver fluke) within infrapopulations from cattle and sheep. *Parasitology Research* **101**, 117–125. doi: 10.1007/s00436-006-0440-4.

Wilkinson, R., Law, C. J., Hoey, E. M., Fairweather, I., Brennan, G. P. and Trudgett, A. (2012). An amino acid substitution in *Fasciola hepatica* P-glycoprotein from triclabendazole-resistant and triclabendazole-susceptible populations. *Molecular and Biochemical Parasitology* **186**, 69–72. doi: 10.1016/j.molbiopara.2012.08.008.

Winkelhagen, A. J., Mank, T., de Vries, P. J. and Soetekouw, R. (2012). Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. *Emerging Infectious Diseases* **18**, 1028–1029. doi: 10.3201/eid1806.120302.

Xu, M. J., Ai, L., Fu, J. H., Nisbet, A. J., Liu, Q. Y., Chen, M. X., Zhou, D. H. and Zhu, X. Q. (2012). Comparative characterisation of microRNAs from the liver flukes *Fasciola gigantica* and *F. hepatica*. *PLoS ONE* **7**, e53387. doi:10.1371/journal.pone.0053387.

Young, N. D., Halls, R. S., Jex, A. R., Cantacessi, C. and Gasser, R. B. (2010). Elucidating the transcriptome of *Fasciola hepatica* – a key to fundamental and biotechnological discoveries for a neglected parasite. *Biotechnology Advances* **28**, 222–231. doi: 10.1016/j.biotechadv.2009.12.003.

Young, N. D., Jex, A. R., Cantacessi, C., Halls, R. S., Campbell, B. E., Spithill, T. W., Tangkawattana, S., Tangkawattana, P., Laha, T. and Gasser, R. B. (2011). A portrait of the transcriptome of the neglected trematode, *Fasciola gigantica* – biological and biotechnological implications. *PLoS Neglected Tropical Diseases* **5**, e1004. doi: 10.1371/journal.pntd.0001004.

https://doi.org/10.1017/S0031182013000528 Published online by Cambridge University Press