

A study of the proteomics of fasciolosis

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Abstract

Fasciolosis is an economically important veterinary parasitic disease. Of the two causative agents, *Fasciola hepatica* and *Fasciola gigantica*, *F. hepatica* has a greater ability to effectively establish a primary infection and resist the host defences. Certain host species, such as cattle, are more resistant to reinfection than others, such as sheep. In order to gain a greater understanding of the host response to infection, proteomic analyses of sera from sheep and cattle infected with *F. hepatica* were undertaken. Twenty-six indicators of infection were validated by SELDI-TOF mass spectrometry (MS) in sheep with a single-dose *F. hepatica* infection. Two of these markers were identified and their increase in the chronic stage of infection was validated using Western blot analysis. SELDI-TOF MS profiling of sera from trickle-infected cattle did not provide descriptive information, but tandem MS analysis of immunodepleted sera provided a more descriptive view of the host-parasite interaction. This technique was sufficiently sensitive to detect markers of inflammation during the acute stage of infection and markers of liver fibrosis during the chronic stage of infection, but was not sufficiently sensitive to detect parasite proteins. Finally, the excretory-secretory products (ESP) of *F. hepatica* and *F. gigantica* were profiled. The similarities between the two species were greater than the similarities between newly excysted juveniles (NEJ) and adults. The most notable differences between NEJ and adults were the profile of proteases released and the specific isotypes released by the parasites. NEJ expressed relatively equal amounts of cathepsin L, B, and legumain, whereas adults expressed predominantly cathepsin L. NEJ- and adult-specific cathepsin L clades were identified as well as a potential *F. gigantica* NEJ-specific cathepsin L clade. Antioxidant defence enzyme levels were higher in adult ESP than NEJ ESP, with higher relative abundance in *F. gigantica* than *F. hepatica*. The analysis suggests that stage-specific expression and isotype diversity should be considered when developing a vaccine targeted to the early stage of infection and when studying broad-spectrum chemotherapeutic targets. These studies contribute to the understanding of the basic biology of the causative

agents of fasciolosis and the use of proteomics in studying host-parasite interactions.

Abrégé

La fasciolase est une infection vétérinaire importante. Entre les deux agents causals, *Fasciola hepatica* et *Fasciola gigantica*, *f. hepatica* a une meilleure capacité pour établir une infection primaire et pour résister aux défenses de l'hôte. Certaines espèces hôtes, tels que les bovins, résistent une seconde infection mieux que d'autres espèces, tels que les moutons. Afin de mieux comprendre la réaction de l'hôte pendant l'infection, des analyses protéomiques de sérums de moutons et de bovins affectés par *f. hepatica* ont été entreprises. Vint-six marqueurs d'infection ont été validés par spectrométrie de masse de temps de vol à désorption-ionisation laser potentialisée par surface (SM TDV-DILPS) chez les moutons avec une seule dose infectieuse de *f. hepatica*. Deux de ces marqueurs ont été identifiés et leur hausse dans la phase chronique de l'infection a été validée par buvardage de western. Le profil SM TDV-DILPS de bovins infectés à plusieurs reprises n'a pas fourni des détails descriptifs, mais une analyse SM en tandem de sérums immunodéplétés a fourni un portrait descriptif de l'interaction hôte-parasite. Cette technique était suffisamment sensible pour détecter des marqueurs d'inflammation pendant la phase aiguë de l'infection et des marqueurs de la fibrose hépatique pendant la phase chronique de l'infection, mais n'était pas suffisamment sensible pour détecter des protéines parasitaires. Enfin, les produits d'excrétion-sécrétion (PES) de *f. hepatica* et *f. gigantica* ont été profilés. Les deux espèces partageaient plus de similitudes que parmi les phases de juvéniles nouvellement excystés (JNV) et des adultes. Les différences les plus notables entre les JNV et les adultes étaient le profil des protéases et les isotypes de ces protéases retrouvés. Les montants de cathepsine L, B et de legumain chez les JNV étaient relativement égaux, tandis que la majorité des protéases chez les adultes étaient cathepsine L. Des clades de cathepsine L spécifiques aux JNV et aux adultes ont été identifiés ainsi qu'un clade potentiellement spécifique aux JNV de *f. gigantica*. Les niveaux d'enzymes antioxydants de défense dans les PES étaient plus élevés dans chez les adultes que les JNV, ainsi qu'une abondance plus élevées chez *f. gigantica* que *f. hepatica*. Ces analyses suggèrent que l'expression

des protéines spécifique aux phases de développement et la diversité des isotypes devraient être considérées lors de l'élaboration d'un vaccin ciblé à un stade précoce de l'infection et lors du développement de cibles chimiothérapeutiques à large spectre. Ces études contribuent aux connaissances fondamentales des agents causals de fasciolase et l'usage d'outils protéomiques dans l'étude des interactions hôte-parasite.

Abbreviations used

1-DE	- one-dimensional gel eletrophoresis
2-DE	- two-dimensional gel eletrophoresis
ACN	- acetonitrile
Apo A-IV	- apolipoprotein A-IV
AST	- aspartate aminotransferase
CatB or CB	- cathepsin B
CatL or CL	- cathepsin L
CHCA	- α -cyano-4-hydroxy-cinnamic acid
CHAPS	- 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CL	- cathepsin L
CV	- coefficient of variation
DIGE	- differential in-gel eletrophoresis
ELISA	- enzyme-linked immunosorbent assay
ES or ESP	- excretory-secretory products
FABP	- fatty acid-binding protein
Fg or Fh	- <i>Fasciola gigantica</i> or <i>Fasciola hepatica</i>
GGT	- γ -glutamyl transferase
GLDH	- glutamate dehydrogenase
GST	- glutathione s-transferase
Hb	- haemoglobin
HEPES	- 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	- high performance liquid chromatography
ITT	- Indonesian Thin Tail
LAP	- leucine aminopeptidase
LC-MS/MS	- liquid-chromatography tandem mass spectrometry
LPS	- lipopolysaccharide
MALDI-TOF	- matrix-assisted laser desorption-ionisation time-of-flight

MS	mass spectrometry
MARS	- multiple affinity removal system
MS	- mass spectrometry
MS/MS	- tandem mass spectrometry
MWCO	- molecular weight cutoff
M/z	- mass-to-charge ratio
NEJ	- newly excysted juveniles
PBS	- phosphate-buffered saline
PCR	- polymerase chain reaction
PDI	- protein disulphide isomerise
p.i. or w.p.i.	- post-infection or week(s) post-infection
PMF	- peptide mass fingerprinting
ROC	- receiver operator characteristic
ROS	- reactive oxygen species
RT	- room temperature
SAP	- saposin
SDS-PAGE	- sodium dodecyl sulphate polyacrylamide gel eletrophoresis
SELDI-TOF	- surface-enhanced laser desorption-ionisation time-of-flight
MS	mass spectrometry
S/N	- signal-to-noise ratio
SOD	- superoxide dismutase
SPA	- sinapinic acid
TCA	- trichloroacetic acid
TFA	- trifluoroacetic acid
TLR	- toll-like receptor
TrxR	- thioredoxin reductase

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Contribution of authors

Chapter 3: Discovery and validation of serum biomarkers expressed over the first twelve weeks of *F. hepatica* infection in sheep.

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M.-C.R. performed the bulk of this study, prepared and edited the manuscript. C.C. and D.A. provided sheep sera, collected in a vaccine trial performed by their group. B.W. and M.N. provided access to Biomek workstation and SELDI-TOF MS, and provided technical training. B.F.G. and H.B. provided MS/MS analyses of Apo A-IV protein. T.W.S. supervised the study and edited the manuscript.

Chapter 4: Comparative proteomic analysis of newly excysted juvenile and adult excretory-secretory products from *Fasciola hepatica* and *Fasciola gigantica*

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Chapter 5: Analysis of the serum proteome of *F. hepatica* trickle-infected cattle

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M.-C.R. performed the bulk of this study, prepared and edited the manuscript. B.W. and M.N. provided access to Biomek workstation and SELDI-TOF MS. T.G. supervised the study and edited the manuscript. T.W.S. supervised the study and edited the manuscript.

Claims of originality

1. Chapter 3 describes the first proteomic profile of *F. hepatica*-infected sera in sheep using SELDI-TOF MS.
2. Chapter 3 includes the first published study to validate markers of parasitic infection identified by SELDI-TOF MS using an alternate method.
3. Chapter 4 is the first comprehensive analysis of *F. gigantica* NEJ ES and adult ES.
4. Chapter 4 includes the first comprehensive comparison of the proteins expressed in *F. hepatica* and *F. gigantica* NEJ and adults
5. Chapter 4 includes the first comparative analysis of cathepsin L clades expressed in both *F. hepatica* and *F. gigantica* NEJ and adults.
6. Chapter 5 reports the first proteomic profiling of sera from *F. hepatica*-infected cattle using SELDI-TOF MS and comprehensive LC-MS/MS analysis.
7. Chapter 5 demonstrates the effectiveness of human IgY-12 column on the heterologous proteins in cattle sera.
8. Chapter 5 includes the identification of markers of inflammation and liver fibrosis in *F. hepatica*-infected cattle using a non-biased approach.

Chapter 1: Introduction and Thesis objectives

Introduction

Fasciola hepatica and *Fasciola gigantica* are veterinary parasites that have an important economic impact in both the North and South (Hillyer and Apt, 1997b; Spithill et al., 1999). The current control methods are centered on regular chemotherapy (McManus and Dalton, 2006). The most commonly used drug is triclabendazole, a narrow-spectrum benzimidazole that is effective against the migrating and adult stages of *F. hepatica* and *F. gigantica*. Regular treatment of livestock with triclabendazole prevents permanent damage associated with migration of the parasite through the liver (Brennan et al., 2007). Resistance is emerging to triclabendazole (Moll et al., 2000) and research into the mechanism of host resistance to infection and vaccine development have come to the forefront, including cooperative consortiums, such as the DeLiver project (www.deliver-project.eu). The following aspects of the host-parasite interaction are important in vaccine development for fasciolosis: the antigens released by the parasite over the life cycle in the definitive host, the host response to the parasite, the modulation of the host response by the parasite, and the differences between the two aetiological agents of fasciolosis.

Fasciola spp. parasites have blind-ended guts that are emptied periodically via the mouth. The products that are released are known as excretory-secretory products (ESP). These proteins are thought to be important for nutrient acquisition by the parasite (Sajid and McKerrow, 2002), and have also been implicated in the modulation of the immune response from a protective T_H1-type response to a non-protective T_H2-type response (O'Neill et al., 2000). The characterisation of these antigens is key to developing successful vaccination strategies, but has been limited by the availability of *Fasciola spp.* genome sequences and the availability of mass spectrometry tools optimized for protein identification. Recent advances in mass spectrometry and bioinformatics has allowed researchers to identify *F. hepatica* ESP proteins (Jefferies et al., 2001; Morphew et al., 2011; Morphew

et al., 2007; Robinson et al., 2009) and has expanded the understanding of the parasite's physiology. Identifying the proteins released by parasites at different stages of the life cycle provides a solid foundation for the identification of effective vaccine candidates.

There are two important issues when studying the host-parasite interaction in fasciolosis: *F. hepatica* and *F. gigantica* differ in their ability to infect hosts, and hosts differ in their ability to effectively respond and control *Fasciola spp.* infections. *F. hepatica* successfully establishes a primary infection in definitive hosts, such as sheep and cattle, but the resistance to a second exposure depends on the host. Cattle, for example, can successfully respond to a second *F. hepatica* infection, whereas sheep cannot (Haroun and Hillyer, 1986). Resistance to primary and secondary *F. gigantica* infection is more widespread. Javanese Indonesian Thin Tail sheep are more resistant to *F. gigantica* infection than other sheep (Wiedosari and Copeman, 1990), while they are susceptible to *F. hepatica* primary and secondary infections. Merino sheep, on the other hand, demonstrate resistance only to secondary *F. gigantica* infections (Roberts et al., 1997a; Roberts et al., 1996). Understanding the differences in antigenic profiles of the two species can identify key proteins responsible for the success of *F. hepatica* and provide rational vaccine candidates. Understanding the differences in the host responses to the pathogen could also identify the type of adjuvant that should be used for successful vaccination and provide indicators that could be used to monitor animals during vaccination trials.

To date, no vaccine candidate has completely protected animals from reinfection with *F. hepatica* (McManus and Dalton, 2006). Vercruysse and Claerebout (2001) have suggested that reducing the impact of disease on the veterinary sector could be achieved by reducing the worm burden below a minimal threshold of ≤ 30 flukes in cattle and herd prevalence of $\leq 25\%$ for chemotherapeutic treatment would represent minimal or no interference in the productivity of cattle. *Fasciola spp.* parasites undergo a complex life cycle within the host and the antigens that are released vary during the development of the parasite (Cancela et al., 2008; Robinson et al., 2009). The parasite first excysts in

the small intestine within the first few hours of ingestion; this newly excysted juvenile (NEJ) then migrates to the liver by traveling across the surface of the peritoneum. Juvenile parasites then penetrate the liver capsule within a few days and feed on the liver parenchyma (Andrews, 1999) causing extensive inflammation and tissue damage. The parasite migrates to the bile duct within 8-16 weeks, depending on the parasite and host species. Minimizing the impact of fasciolosis on animals can be accomplished by stimulating an immune response to the infection before the liver damage becomes irreversible: therefore antigens from the newly excysted and juvenile stages should be the prime focus of vaccine studies. Recent phylogenetic studies combined with mass spectrometry data suggest that cathepsin L clades 3 and 4 are unique to the NEJ stage and may represent new vaccine candidates to provide protection against the early migratory phase of infection (Cancela et al., 2008; Robinson et al., 2009).

There is evidence that *Fasciola spp.* modulate the immune response from the more effective T_H1-type response to a less effective T_H2 response in mice (O'Neill et al., 2000), sheep (Raadsma et al., 2007) and cattle (Mulcahy et al., 1998). Certain ESP molecules have been linked directly to host immunosuppression, including cathepsin L1 (Donnelly et al., 2010; Dowling et al., 2010), sigma-class glutathione transferase (Dowling et al., 2010), thioredoxin peroxidase (Donnelly et al., 2005), peroxiredoxin (Donnelly et al., 2008), and tegumental proteins (Hamilton et al., 2009). Antigens that account for the increased virulence of *F. hepatica* would be ideal vaccine candidates, since neutralizing these antigens would permit the animal to mount an effective immune response to infection.

Integrating knowledge from each of these aspects of the interaction of *Fasciola spp.* parasites with its hosts will help guide the selection of antigens in vaccine development and monitor the development of productive responses in sheep and cattle in order to reduce the burden of fasciolosis in both the North and South.

Thesis objectives

1. The first aim of this thesis is to gain a greater understanding of the host response to *F. hepatica* infection in sheep through the following objectives:
 - a. Profile serum proteins of experimentally infected Merino sheep using SELDI-TOF MS and identify statistically significant markers of infection.
 - b. Identify the biomarkers of infection and validate using an alternative methodology.
2. The second aim of this thesis is to gain a greater understanding of the host response to *F. hepatica* infection in cattle through the following objectives:
 - a. Profile serum proteins of cattle experimentally infected with *F. hepatica* using SELDI-TOF MS and identify statistically significant markers of infection.
 - b. Identify markers of infection by LC-MS/MS based technology using depleted cattle sera.
3. The third aim of this thesis is to profile excretory-secretory products (ESP) from *F. hepatica* and *F. gigantica* NEJ and adults to identify potential markers of virulence through the following objectives:
 - a. Collect ESP samples *in vitro* and analyse the proteins by LC-MS/MS
 - b. Carry out bioinformatic analysis to identify potential virulence or susceptibility markers

Fulfillment of the first two objectives will give a greater understanding of the response to *F. hepatica* infection in sheep and cattle. The final objective will provide an overview of the proteins released by *Fasciola spp.* parasites and a basis for comparison of the virulence of these two species, which will ultimately aid in the selection of vaccine candidates for a vaccine that has broad specificity and stimulates an early response to infection.

Chapter 1 References

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Chapter 2: Literature review

1. Fasciolosis

1.1. Aetiology of fasciolosis and geographic distribution

Fasciolosis is an infection of herbivores and humans caused primarily by the digenean trematodes *Fasciola hepatica* and *Fasciola gigantica*. *F. hepatica* is found in temperate climates and is widely distributed worldwide, whereas *F. gigantica* is found in tropical climates with a much more focal distribution (Mas-Coma et al., 2005). *F. hepatica* infections are economically important in sheep and cattle, but can also affect a wide range of susceptible hosts, including goats, horses and pigs. Other hosts include llamas in South America, camels in Africa and marsupials in Australia (McManus and Dalton, 2006). *F. gigantica* infections are particularly important in cattle and water buffalo in Asia (McManus and Dalton, 2006) and regionally can be an important infection of goats, sheep, and donkeys (Spithill et al., 1999). The distribution of *F. hepatica* and *F. gigantica* overlap in regions where fasciolosis is important, including Japan, Korea, Taiwan and the Phillipines. This has given rise to mixed parasite forms by apparently natural hybridisation (Agatsuma et al., 2000; Le et al., 2008). Much attention has been focused on the veterinary impact of fasciolosis, yet it has an important impact on humans as well. Human infections have been reported in 51 countries from five continents, representing a geographic distribution as widespread as for veterinary fasciolosis. Fasciolosis is not only a sporadic disease associated with animal infections, but is truly endemic, affecting principally children and females (reviewed in Mas-Coma et al., 2009).

1.2. Life cycle

Fasciola spp. parasites have a digenean life cycle in that they undergo a round of sexual replication in the definitive mammalian host, and a round of asexual replication in the intermediate snail host, as represented in Figure 1 below. The definitive hosts of *Fasciola spp.*, including humans and ruminants such as sheep, cattle, and buffalo, become infected when they ingest water or food

contaminated with encysted metacercariae. Metacercariae enter the digestive tract and excyst in the small intestine within a few hours of ingestion. The newly excysted juveniles (NEJ) preferentially migrate towards the liver by penetrating the mucosa of the small intestine, breaking through epithelial cells, connective tissue and unstriated muscle fibres and then travel across the surface of the peritoneum. Juvenile flukes penetrate the liver capsule within a few days and cause inflammation as the growing parasites feed on liver parenchyma (Andrews, 1999).

In *F. hepatica* infections, the parasite enters the bile duct approximately seven to ten weeks after infection, where it matures to adulthood and begins to produce eggs about 8-10 weeks post-infection in sheep. The prepatency period of *F. gigantica* infections in mammals is longer, estimated at 13-16 weeks in sheep and cattle (Wiedosari and Copeman, 1990; Yadav et al., 1999; Zhang et al., 2004a). The parasite eggs are released into the large intestine by bile fluids and are passed onto pasture by faeces. *Fasciola spp.* have been estimated to produce 20 000 to 50 000 eggs per fluke per day (Boray, 1969). The miracidium hatches from the eggs and infects mud-snail hosts such as *Lymnae truncatula*. The parasite undergoes a series of asexual replicative stages and emerges from the snail to encyst on waterborne vegetation or on the water surface (Andrews, 1999).

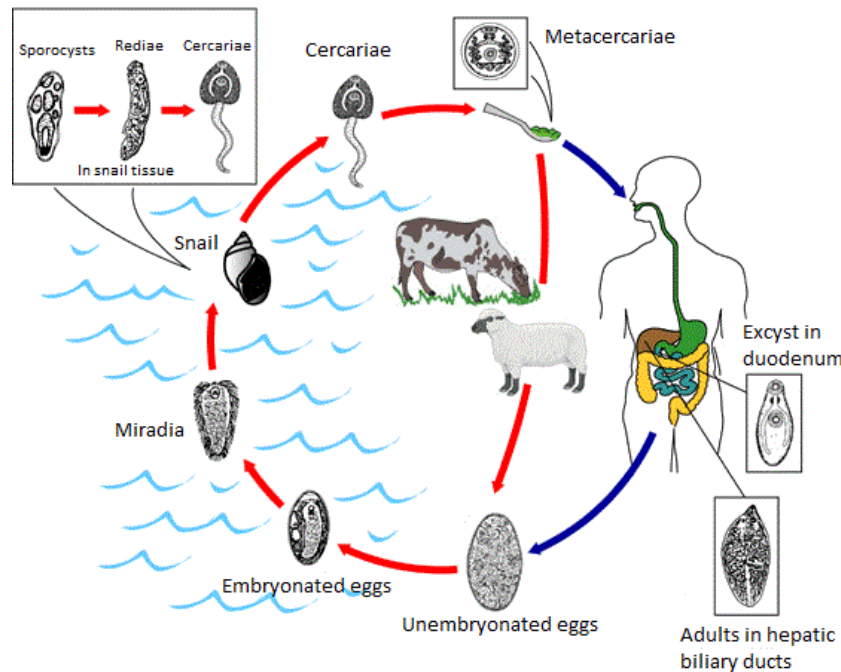


Figure 1: Life cycle of *Fasciola* spp. (adapted from CDC, 2009)

1.3 Anatomy of *Fasciola* spp. in the definitive host

The adult fluke has a flattened leaf shape and has muscular oral and ventral suckers at the anterior end. The suckers are important for migratory movement, feeding and attachment. The surface of the parasite is covered by spines, made up largely of actin, and sensory papillae. *Fasciola* spp. have three main strata: the outer tegument, the underlying musculature, composed of outer circular muscles and inner longitudinal muscles, and the parenchyma (Fairweather et al., 1999).

The tegument is the outer layer of the parasite and is in direct contact with the host. It is a syncytial layer that is connected to the parenchyma by cytoplasmic extensions that cross the intervening muscle layers. The tegument has both secretory and absorptive functions. The tegument is also responsible for protection from host digestive enzymes, bile and effectors of the immune response. The tegument has secretory bodies that change as the fluke matures. In the newly excysted juveniles (NEJ), the secretory bodies are T0 bodies, which become T1 bodies as the parasites mature into juveniles. T2 bodies begin to form

within one week of encystment. Adult tegument contains only T1 and T2 bodies (Bennett and Threadgold, 1975). The tegument is covered by a glycocalyx that is continuously replaced in early developmental stages, helping slough off attached host antibodies and thus contributing to evasion of the host immune response (Fairweather et al., 1999).

The parenchymal cells fill the spaces between the organ systems in the fluke. In the absence of a circulatory system, the parenchyma transports substances around the body of the fluke (Gallagher and Threadgold, 1967; Threadgold and Gallagher, 1966). The parenchyma is also associated with carbohydrate metabolism, including glycogen storage in NEJ (Bennett, 1977; Bennett and Threadgold, 1973) and adults (Threadgold and Arme, 1974).

NEJ and adult fluke musculature have identical structure in the sucker and somatic regions, consisting of invertebrate smooth type muscle (Bennett and Threadgold, 1973). A paramyosin-like protein, a component of the thick filaments in some invertebrates, has been extracted from fluke muscle and; the molecular mass (98 kDa) and amino acid composition are similar to those of other invertebrates (Ishii and Sano, 1980). There is evidence that there are Ca^{2+} pumps on the sarcolemma (Skuce, 1987).

Fasciola spp. are able to detect environmental cues and respond accordingly because of nervous system communication. The central nervous system consists of a pair of anterior ganglia located just posterior to the oral sucker, which are connected through a transverse commissure. The peripheral nervous system is composed of cell bodies and nerve fibres beneath the tegument, in the oral and ventral suckers and in the reproductive organs. Several neurotransmitters are present in the fluke, including acetylcholine, serotonin, dopamine and neopinephrine (Fairweather et al., 1999).

Fasciola spp. adults have blind guts that are emptied approximately every three hours (McManus and Dalton, 2006). The cells lining the caeca undergo cyclical transformations, varying between absorptive and secretory phases (Fairweather et al., 1999). In NEJ, the cells lining the caeca are specialized for secretion only, and contain vesicles that are produced and stored during the

metacercarial phase. In adults, cells are either in a secretory phase, absorptive phase, or a third form that is associated with the movement of material back and forth within the lumen of the main caeca and with the mixing of secretions with the material (Robinson and Threadgold, 1975). The parasites digest material extracellularly by releasing enzymes into the lumen of the caeca and reabsorbing digested material. The diet of the parasite varies as the parasite matures, initially composed of abdominal viscera in the migratory phase, hepatic cells and some blood in the liver phase, and primarily blood in the bile duct phase. Enzymes released into the gut lumen include proteolytic enzymes such as legumain and cathepsins B and L. The caeca contain both the secreted materials as well as excretory materials, which are released into the surroundings through the oral pore periodically. The materials released are excretory-secretory products (ESP) and have been studied extensively to determine their role in the host response to the parasite as well as the immunomodulation of the parasite. Components of ESP have been associated with digestion, tissue destruction, and immune evasion.

1.4 Ruminant host pathology

Fasciolosis consists of two phases: the parenchymal phase during the migration of flukes through the liver parenchyma, and the biliary phase, which coincides with parasite establishment within the bile ducts (Behm and Sangster, 1999). The parenchymal phase of the disease is characterised by anaemia due to the mechanical damage caused by the spines and host sucker of the parasite, as well as the direct feeding of the parasite at the rate of 0.2-0.5 mL blood per day per fluke (Dawes and Hughes, 1964). A cellular inflammatory reaction to the mechanical damage in the liver then occurs (Dow et al., 1967, 1968; Ross et al., 1967). The tracks fill with cell debris, erythrocytes, lymphocytes, neutrophils, eosinophils and macrophages and damage to the hepatic cells surrounding is seen (Dow et al., 1968; Meeusen et al., 1995; Ross et al., 1966). The infiltrating lymphocytes are predominantly CD4⁺ T cells, with less CD8⁺ T cells and few B cells and T19⁺ cells (Meeusen et al., 1995). Older parts of the tracks gradually fill with macrophages and fibrosis may occur in certain hosts (Ross et al., 1966; Dow

et al., 1968). The lymphocytes in the chronic stage have a significantly higher proportion of CD8 to CD4 T cells and the presence of $\gamma\delta$ -TCR⁺ T cells in the fibrotic areas (Meeusen et al., 1995).

Glutamate dehydrogenase (GLDH) (EC 1.4.1.2) activity in the serum is used as a marker of parenchymal tissue damage during the acute phase of infection. Increased GLDH levels are seen as early as two weeks after infection (Anderson et al., 1977) with maximum levels coinciding with the end of the parenchymal phase, at about week 13 post-infection (Anderson et al., 1981; Sykes et al., 1980). Serum aspartate aminotransferase (AST) (EC 2.6.1.1) activity, associated with pathology to the liver parenchyma or skeletal and cardiac muscle, has also been used to monitor parenchymal tissue damage, but is not sufficiently specific as increases may also be attributed to skeletal muscle trauma caused by animal handling during the course of the experiment (Bulgin et al., 1984; Wyckoff and Bradley, 1985).

As the parasites enter the bile duct, bile duct damage is signalled by an increase in γ -glutamyl transferase (GGT) (EC 2.3.2.2) activity in the serum while the inflammation of the parenchymal tissue subsides. The peak of GGT activity follows the peak of the GLDH and AST (Bulgin et al., 1984). In the biliary stage, in the absence of concurrent parenchymal damage, GGT levels fall but remain higher than in uninfected controls. Anaemia is also characteristic of the bile duct phase of the *Fasciola spp.* life cycle. There is a positive relationship between the total daily blood loss and the number and age of flukes recovered, but the blood loss per fluke declines with high fluke burdens (Sinclair, 1972). Chronic disease can persist for several years in cattle and many years (as long as 20) in sheep.

Peripheral eosinophilia is a marker of infection throughout the course of *Fasciola* infection; it increases throughout the parenchymal phase and persists through the biliary phase in all hosts (Ross et al., 1966).

1.5 Impact on cattle and sheep

Bovine and ovine fasciolosis cause direct economic losses with an estimated 250 million sheep and 350 million cattle at risk (Hillyer and Apt,

1997b). Losses due to fasciolosis are estimated at over 3 billion USD annually (Spithill et al., 1999). These losses include reduction in meat and milk production, as well as diminished quality of wool in sheep and alpacas, condemned livers, abortions and fertility reduction.

Sheep are often exposed to large inocula of *Fasciola spp.* when local environmental conditions are conducive for high and persistent rates of transmission, sometimes taking the form of outbreaks of acute disease with high mortalities (Haroun and Hillyer, 1986). In addition, sub-lethal doses of *F. hepatica* infection in sheep have been associated with anaemia (Sinclair, 1972), loss of appetite (Ferre et al., 1994; Hope Cawdery et al., 1977), weight loss (Hope Cawdery et al., 1977; Sykes et al., 1980), reduction in wool production (Roseby, 1970) and reduced fertility (Charlier et al., 2007; Hope Cawdery, 1976). Hawkins (1984) observed that the pathology of the disease associated with *F. hepatica* is not completely reversible by drug treatment. The study demonstrated that unless the sheep were treated with diamphenethide within four weeks of infection, no compensatory growth occurred following chemotherapy and sheep demonstrated a persistent reduction in productivity, despite normal food intake after treatment. Raadsma et al. (2007) found that high inocula of both *F. hepatica* and *F. gigantica* induced similar effects on production in sheep and directly influenced weight gain within 2 weeks post-infection, despite the greater damage caused by *F. hepatica* infection based on fluke biomass and liver damage. The authors suggest that there is a parasite threshold above which production losses are comparable in the two species.

Although lethal outbreaks are uncommon among cattle, modest fluke infections can result in significant reductions in performance, including weight gain, fertility and milk production. Infections in cattle are widespread, with the prevalence rates in cattle estimated at 18% Switzerland (Rapsch et al., 2006), 39%-78% in Vietnam (Anderson et al., 1999; Geurden et al., 2008), 48% in England and 86% in Wales (Salimi-Bejestani et al., 2005). The greatest reduction in weight gain in young cattle occurs in the first 16 weeks of infection and a significant decrease in weight gain persists during the chronic stage of the disease.

Even after the animals are cleared of flukes, the initial impaired performance remains until slaughter (Hope Cawderly and Conway, 1971). Losses in milk yield of 0.7 kg per day per cow have been observed in large farm survey in Belgium (Charlier et al., 2007).

1.6 Human impact

Fasciolosis is increasingly being recognized as a disease of clinical importance, with an estimated 2.4 million people infected with *Fasciola* species and 180 million people at risk of infection (WHO, 1995). Fasciolosis in humans has most often been described as being caused by *F. hepatica*, but has also been found to be caused by *F. gigantica* (Hammond, 1974). Cases of human fasciolosis have been largely under-reported, with less than 3000 *F. hepatica* infections in 42 countries, from Europe, Latin America, North Africa, Asia and the western Pacific cited over the course of 25 years by the World Health Organization (Chen and Mott, 1990). Recent calls have been made to establish a baseline assessment of the epidemiology of human fasciolosis given the lack of knowledge about the situation in many African and Asian countries in order to understand the factors involved in managing this neglected disease in human populations (Mas-Coma et al., 2009).

Recent studies have shown that human fasciolosis is endemic in several areas, including the high altitude Andean rural regions stretching from northern Bolivia, through Peru to Ecuador (Mas-Coma et al., 1999a; Mas-Coma et al., 1999b), in the Guilan and Mazandaran provinces of northern Iran (Moghaddam et al., 2004; Rokni et al., 2002) and the Nile Delta region in Egypt (Esteban et al., 2003). There have also been case reports from Vietnam (Xuan le et al., 2005), Malaysia (Naresh et al., 2006), and Turkey (Saba et al., 2004). Outbreaks have been reported in developed countries including France, Portugal, Spain and Australia (Mas-Coma, 2005). Diagnoses are increasingly relying on eosinophilia and serological findings, including the use of a specific and sensitive IgG₄ subtype-specific cathepsin L1 enzyme-based ELISA assay (O'Neill et al., 1999). This technique has been used in prevalence studies in the Bolivian Altiplano,

where *F. hepatica* is endemic (Mas-Coma et al., 1999b). Prevalence was estimated to be 21% (O'Neill et al., 1998). Infection is found mainly in indigenous peoples, subsistence farmers who regularly share the same water sources with their animals. Infection is more prevalent in children but is present in all ages of both sexes (Mas-Coma, 2005).

1.7 Diagnosis

The current diagnostic gold-standard for humans in the use of cathepsin L1 (CL1) in an indirect ELISA assay specific for IgG₄ antibody isotype. The ELISA using CL1 recombinant protein has been found to be both sensitive and specific for fasciolosis caused by *F. hepatica* (Carnevale et al., 2001; O'Neill et al., 1999; O'Neill et al., 1998; Strauss et al., 1999) as well as *F. gigantica* (Tantrawatpan et al., 2005; Wongkham et al., 2005), as has a CL1 peptide-based ELISA for the diagnosis of *F. gigantica* (Intapan et al., 2005; Tantrawatpan et al., 2007). Testing in animals relies on less invasive assays, including ELISA of milk and coproscopical analyses, though serum-based ELISAs using CL1 have been found to be effective in cattle (Cornelissen et al., 1999) and sheep (Carnevale et al., 2001; Wijffels et al., 1994b). A commercially available ELISA test is effective for the diagnosis of *F. hepatica* infection using sera and milk from sheep and cattle, as well as the diagnosis of *F. gigantica* using sera from buffalo and cattle (Molloy et al., 2005). The use of purified cysteine proteases has been shown to be effective in indirect ELISAs using goat serum (Ruiz et al., 2003). Using *F. hepatica* excretory-secretory products (ESP) in ELISA testing, the ELISA OD value has been found to be positively correlated with intensity of infection in cattle (Salimi-Bejestani et al., 2008). A monoclonal antibody specific for a *F. hepatica* ES antigen, called MM3, has been used in capture ELISA to detect *F. hepatica* antigen in fecal supernatant from sheep and cattle infected with *F. hepatica* with good sensitivity (Mezo et al., 2004). Recent work has also shown the MM3-ELISA is also effective at detecting *F. gigantica* infection in sheep (Valero et al., 2009). The testing of the MM3-ELISA was expanded to include human stool samples preserved with CoproGuard from individuals, and was found to be

sensitive with no cross-reactivity with other common parasites (Ubeira et al., 2009). This approach to coproantigen testing is a promising approach to non-invasive fasciolosis testing in animals as well as humans.

1.8 Control

Control of infection among human populations relies on targeted chemotherapeutic intervention in communities with high prevalence. Human fasciolosis is treated with a single dose of triclabendazole (10 mg per kg of body weight) (WHO, 1998), which replaced the previous treatment with bithionol. Curtale et al. (2005) described the successful first attempt to control human fasciolosis using triclabendazole in a school-based intervention strategy implemented in the Nile Delta, Egypt.

Chemotherapy is the centre of the current veterinary fasciolosis control strategy in Europe and Australia (McManus and Dalton, 2006). Common fasciolicides either recently used or commonly used today include halogenated phenols (e.g. bithionol), salicylanilides (e.g. closantel), benzimidazoles (including triclabendazole), and sulphonamides (e.g. clorsulon) (Fairweather and Boray, 1999). Most fasciolicides, including bithionol, closantel and clorsulon are effective only against the adult stage of the parasite, whereas triclabendazole is effective against both the immature and adult stages of the parasite. Triclabendazole (Fasinex[®], Novartis) is a narrow-spectrum benzimidazole that is effective against *F. hepatica*, *F. gigantica* and *Fascioloides magna* and shows greater efficacy against immature parasites (Fairweather and Boray, 1999). Triclabendazole is the most commonly used drug for the control of fasciolosis in livestock as it is able to prevent the permanent damage associated with migration of immature flukes through the liver parenchyma (Brennan et al., 2007). Triclabendazole is thought to bind to tubulin, therefore interfering with microtubule-based processes, and also interferes with protein synthesis (Fairweather and Boray, 1999). Resistance of liver flukes to triclabendazole in naturally infected sheep has been reported in Australia (Overend and Bowen, 1995), Ireland (Lane, 1998), Wales (Thomas et al., 2000), Scotland (Mitchell et

al., 1998) and Spain (Alvarez-Sanchez et al., 2006). Resistance has also been observed on a farm with cattle and sheep in the Netherlands (Moll et al., 2000). Reduction of treatment efficacy has been demonstrated in controlled *in vivo* experiments in sheep for Danish (Gaasenbeek et al., 2001), Irish (Coles et al., 2000; Coles and Stafford, 2001), and Australian isolates (Walker et al., 2004). The best method of preventing the spread of resistance may be to use combinations of fasciolicides (Fairweather and Boray, 1999). The cost of treatment prevents its wide use by rural producers in developing countries (Spithill and Dalton, 1998).

As the incidence of anthelmintic resistance increases, attention focuses on vaccination. The target in the veterinary community is to reduce the economic impact of fasciolosis in the agricultural sector, which can be accomplished by reducing the worm burden below a minimal threshold. Vercruysse and Claerebout (2001) have suggested that a production-based threshold of ≤ 30 *F. hepatica* flukes in cattle or a herd prevalence of $\leq 25\%$ for chemotherapeutic treatment, a level which would represent minimal or no interference in the productivity of cattle (Hope Cawdery et al., 1977). Vaccine candidates, detailed below, have not shown 100% efficacy, but have the potential to reduce the worm burden in individual animals below designated production-based thresholds and also contribute to reducing the prevalence in a herd. Vaccines present an economically and environmentally sustainable alternative to chemotherapy.

1.9 Vaccine candidates

A number of defined proteins from *F. hepatica* and *F. gigantica* are considered vaccine candidates, including fatty acid-binding protein (FABP), glutathione S-transferase (GST), cathepsin L proteinase and haemoglobin (Hb) (Spithill and Dalton, 1998). FABP vaccination has a modest effect on reduction of worm burden in sheep (Lopez-Aban et al., 2007; Lopez-Aban et al., 2008) and buffalo (Nambi et al., 2005), but has also shown significant anti-fecundity effects. Native GST has shown 29% protection overall in sheep (lowest 0% and highest 65%) and 45% in cattle (lowest 19%, highest 69%) with significant decreases in

parasite fecundity (McManus et al., 2006). Cathepsin L proteases have consistently provided protection in cattle that ranges from 38-68% with large reductions in parasite egg production and viability. When combined with a Hb-containing fraction from *F. hepatica* ESP, protection reaches 72% (Dalton et al., 1996). Leucine aminopeptidase has also emerged as a potential vaccine candidate, with 89% protection in sheep (Piacenza et al., 1999). The vaccines do not confer sufficient protection to prevent infection, but reducing the parasite-induced burden sufficiently may reduce production losses associated with parasite infection pathology, as there appears to be a threshold effect (Raadsma et al., 2007). An alternative approach to modulating the immune system to provide protection includes the use of the adjuvant QuilA, which promotes a protective T_H1 response. A recent study has shown that QuilA administration during infection with *F. hepatica* in sheep leads to a significant reduction in fecal egg count and significantly higher parasite-specific serum antibody activity for all isotypes measured, but does not significantly affect the worm burden (Hacariz et al., 2009).

2. Host responses to *F. hepatica* and *F. gigantica* infections

2.1 Resistance to *F. hepatica* secondary infection is host-dependent

Mammalian hosts are reported to vary in their ability to acquire resistance against challenge infection with *F. hepatica* or *F. gigantica* following primary homologous sensitization, as summarized by Haroun and Hillyer (1986). Animals can broadly be categorized into those that do not develop resistance to *F. hepatica* infection as a result of a primary exposure to the pathogen, including sheep and rabbits, and those that are able to resist a second *F. hepatica* infection after a primary exposure, including mice, rats and cattle. There is no evidence to indicate that primary sensitization of sheep with *F. hepatica* stimulates any resistance to challenge in terms of reduction in the number of worms recovered from a second challenge infection (Chauvin et al., 1995; Kelly and Campbell, 1979; Sinclair, 1973). However, other manifestations, such as retarded worm growth (Sinclair, 1971, 1973), decrease in worm size, reduced egg production, delay of onset of

anaemia and elevated antibody titres, can be seen as effects of a primary sensitizing infection.

Cattle acquire resistance to challenge infection with *F. hepatica* when they are sensitized by primary homologous patent or drug-abbreviated homologous infections (Boray, 1969; Doyle, 1971, 1973; Hoyle et al., 2003; Ross et al., 1966). This is usually manifested by a decrease in the size and number of flukes recovered from challenge. Rejection of parasites occurs by 24 weeks after a primary infection and there is a direct relationship between the duration of the primary infection and resistance to challenge (Doy and Hughes, 1984; Doyle, 1973). Studies done by recovering challenge flukes from either the liver or body cavity of cattle indicate that resistance mechanisms against challenge flukes are effective at or soon after penetration of the liver (Doy and Hughes, 1984). Resistance to a challenge infection in cattle appears to involve both the physical barrier of hepatic fibrosis due to primary infection (Boray, 1967; Ross et al., 1967) and specific acquired immune mechanisms.

2.2 Potential mechanism of *F. hepatica* NEJ killing by the resistant host

The mechanism of host killing of *F. hepatica* parasites in secondary infections in resistant animals has best been studied in rats. Davies and Goose (1981) first described the killing of NEJ in previously sensitized rats, where NEJ injected peritoneally were rapidly coated with peritoneal cells, mainly eosinophils. The eosinophils adhered closely to the flukes and degranulated onto their surface, releasing peroxidase. Vacuoles formed in the NEJ tegument and a portion of the parasite were surrounded by phagocytic cells, particularly neutrophils (Davies and Goose, 1981). *F. hepatica* NEJ have been found to be relatively resistant to killing by free radicals as compared to schistosomulae of *Schistosoma mansoni* (Piedrafita et al., 2000), which has been attributed in part to the activity of oxidant scavenger enzymes of NEJ (Piedrafita et al., 2000). Monocytes/macrophage-rich lavage cell populations from rats mediate antibody-dependent cell-mediated cytotoxicity against *F. hepatica* NEJ through the production of nitric oxide

(Piedrafita et al., 2001). Sheep peritoneal and lung lavage cells differed substantially in their ability to generate nitric oxide and were not able to mediate antibody-dependent cell-mediated cytotoxicity against *F. hepatica* NEJ (Piedrafita et al., 2001).

2.3 Resistance to primary and secondary *F. gigantica* infection

As compared to *F. hepatica*, resistance to primary and secondary *F. gigantica* infections is more widespread. Resistance to *F. gigantica* challenge infection has been reported in Merino sheep (Roberts et al., 1996), and goats (Haroun et al., 1989). In addition, Javanese Indonesian Thin Tail (ITT) sheep have been found to be more resistant to *F. gigantica* primary infection compared to other animals, as measured by percentage take of metacercariae and the severity of pathological changes (Hansen et al., 1999; Pleasance et al., 2011b; Roberts et al., 1997b; Wiedosari and Copeman, 1990), while they are susceptible to *F. hepatica* primary and secondary infections (Pleasance et al., 2011a; Roberts et al., 1997a). The main component of the high resistance of ITT sheep against *F. gigantica* is acquired and acts against juvenile parasites; ITT sheep showed resistance within three weeks of infection. The resistance of ITT sheep to *F. gigantica* was suppressed by administration of dexamethasone. The authors suggested that the resistance to primary *F. gigantica*, but not *F. hepatica*, infection may be in an increased response to an antigen released by the parasite or the ability to neutralize an immunological suppressor that is released only by *F. gigantica* (Roberts et al., 1997b). The high resistance of ITT sheep was thought to be genetically determined by a major gene with incomplete dominance (Roberts et al., 1997c) but recent studies have shown that multiple loci are likely involved (Piedrafita et al., 2010).

Possible mechanisms suggested for ITT resistance to *F. gigantica* infection include reduced IgG₂ responses (Hansen et al., 1999), increased expression of superoxide dismutase in *F. hepatica* NEJ as compared to *F. gigantica* NEJ, implicated in protection from antibody-mediated cytotoxic

killing mechanism, and a resident peritoneal cell population capable of killing *F. gigantica* NEJ (Piedrafita et al., 2007).

Previous studies have shown that serum from both ITT and Merino sheep at 12 w.p.i. demonstrate IgM, IgG₁ and IgE responses to *F. gigantica* with similar antigen profiles by Western Blotting, but ITT sheep produce significantly lower levels of IgG₂ when measuring end point titres (Hansen et al., 1999; Raadsma et al., 2007). A recent study by Pleasance et al. (2011b) suggests that there is a higher relative expression of IgG₂ at 3 w.p.i. in *F. gigantica*-infected ITT sheep, an antibody isotype associated with type 1 immune responses, and that this balance shifts at 10 w.p.i. to a higher IgG₁/IgG₂ ratio. A significantly higher IgG₁/IgG₂ ratio was found in *F. hepatica*-infected ITT sheep, indicating that a type 1 antibody response at 3 w.p.i. corresponds to a resistant phenotype. In light of these recent findings, the antibody isotype balance early in infection is most relevant in assessing the resistance of the host to infection.

Piedrafita et al. (2007) demonstrated that peritoneal cells from *F. gigantica*-infected ITT sheep, rich in macrophages and eosinophils, mediated antibody-dependent cytotoxicity against juvenile *F. gigantica* *in vitro*. Cytotoxicity was dependent on contact between the parasite and effector cells and killing was dependent on the production of superoxide radicals by macrophages and eosinophils. This cytotoxic mechanism was ineffective against juvenile *F. hepatica* parasites *in vitro*. Analysis of superoxide dismutase activity and mRNA levels showed that activity and gene expression were higher in *F. hepatica* than in *F. gigantica*, suggesting a possible role for this enzyme in the resistance of *F. hepatica* to superoxide-mediated killing. Peritoneal cells (mainly monocytes/macrophages) from naïve ITT sheep also elicited killing of NEJ *F. gigantica* *in vitro*, suggesting that this ITT sheep cell population is able to act against the invading parasite.

2.4 Differences in *F. hepatica* and *F. gigantica* pathogenesis

Studies have shown that when an equal number of *F. hepatica* or *F. gigantica* metacercariae are administered to ruminants, more flukes are

recovered from the *F. hepatica* infections than the *F. gigantica* infections (Pleasant et al., 2011a). Using fluke biomass, average fluke size, degree of liver damage, plasma GLDH levels and GGT responses, Raadsma et al. (2007) found that *F. hepatica* is more pathogenic than *F. gigantica* in the first 10 weeks of infection due to its faster rate of growth and the speed of its progression through the migratory phases to establishment in the bile duct.

The comparison of the host responses to *F. hepatica* and *F. gigantica* in ITT and Merino sheep has provided insight into the factors associated with resistance and susceptibility to infection. A recent study has characterized the host response to *F. hepatica* infection in ITT sheep and compared it to the response of ITT and Merino sheep to *F. gigantica* infection (Pleasant et al., 2011b). The study demonstrated that ITT sheep differ in their initial response to *F. hepatica* and *F. gigantica* infections: the early response to *F. hepatica* infection is a predominantly type 2 response, whereas the early response to *F. gigantica* infection is a predominantly type 1 response. The type 2 response to *F. hepatica* was characterized by significantly increased IL-4/IFN- γ mRNA ratios in hepatic lymph nodes at 3 and 10 w.p.i. compared to *F. gigantica* infection, increased IL-5 and IL-13 levels at 3 and 10 w.p.i., and reduced IL-2 and IFN- γ levels at 10 w.p.i. The type 2 response to *F. hepatica* was also characterized by increased levels of IL12p40 and IL-2. Additionally, a significantly higher IgG₁/IgG₂ ratio was found in *F. hepatica*- compared to *F. gigantica*-infected ITT sheep at 3 w.p.i., with a decrease in IgG₂ in *F. gigantica*-infected sheep at 10 w.p.i. The cytokine profile of *F. gigantica*-infected Merino sheep, a host susceptible to *F. gigantica* primary infection, showed higher IL-4/IFN- γ mRNA ratio compared to ITT at 3 and 10 w.p.i., suggesting that the type 2 cytokine response is associated with the susceptibility of ITT sheep to *F. hepatica* infection. (Pleasant et al., 2011b). A study of fasciolosis in Bellilois sheep found that the production of IL-10, an inhibitor of the T_H1 response, by FhES-stimulated PBMC is detectable in the first week of infection and is very high throughout infection in *F. hepatica*-infected sheep. In *F. gigantica*-infected sheep, IL-10 production by FgES-stimulated PBMC increased only between 1 and 4 w.p.i., with significantly lower IL-10

production in *F. gigantica*-infected sheep during infection. Higher IFN- γ and lower IL-10 production by FgESP-stimulated PBMC in *F. gigantica* early infection implies an early T_H1/0 type immune response in *F. gigantica*-infected sheep. The authors suggest that the lower susceptibility to *F. gigantica* infection in sheep could be explained by the more intense cellular response induced by the parasite and the weaker capacity of *F. gigantica* to evade the immune response (Zhang et al., 2005b).

There are conflicting reports of the role of eosinophilia in host resistance to infection, with higher eosinophilia correlated with *Fasciola*-susceptible (Roberts et al., 1997a; Waweru et al., 1999) and *Fasciola*-resistant hosts (Hansen et al., 1999; Zhang et al., 2005a). A recent study of *F. hepatica* and *F. gigantica* pathogenesis in naïve ITT sheep showed no significant difference between eosinophil counts during the course of the primary infections and found no correlation between eosinophil counts and the number of parasites recovered (Pleasant et al., 2011b). The animal's cytokine profile may be a much more accurate predictor of disease outcome, as a positive correlation between the GLDH levels (an indicator of liver damage) and IL-4/ IFN- γ mRNA ratio in hepatic lymph nodes was found for both *F. hepatica* and *F. gigantica* infections in ITT sheep (Pleasant et al., 2011b).

3. Proteome of *F. hepatica* and *F. gigantica*

The following is a brief overview of the current state of knowledge of the *Fasciola spp.* proteome.

3.1 Proteases

3.1.1 Cathepsins

Dalton and Hefferman (1989) identified 11 cysteine proteases in ESP released by immature and mature *F. hepatica in vitro* that were active at physiological pH. Using degenerate oligonucleotide amplification of mRNA sequences isolated from adult *F. hepatica*, Heussler and Dobbelaere (1994) confirmed that *Fasciola spp.* encode a family of cathepsin L-like proteases with at

least five different members (termed Fcp1-5). Wijffels et al. (1994a) purified several forms of cysteine proteases in the ESP of adult *F. hepatica*, finding three dominant cysteine proteases that migrated closely in two-dimensional gel electrophoresis and four minor species at a higher isoelectric point (pI), confirming that several forms of cysteine proteases are produced by *Fasciola spp.* Smith et al. (1994) and Dowd et al. (1994) each identified individual proteases from ESP of *F. hepatica* as cathepsin L-like enzymes of 27 kDa (termed CL1) and 29.5 kDa (termed CL2), respectively. These parasite enzymes are unique in that they retain their activity over a wide pH range (4.0-8.5) and are stable at neutral pH, unlike human cathepsin Ls, suggesting a biological function of these enzymes during pathogenesis. Yamasaki et al. (1992) immunolocalized the cysteine protease to the secretory granules of the parasite and found that the protease was secreted as a digestive enzyme into the intestinal lumen, where it was hypothesized to play an important role in the degradation of host proteins, including haemoglobin.

Though most early work focused on analysis of *F. hepatica*, Grams et al. (2001) found at least six different genes encoding cathepsin L in *F. gigantica* by reverse-transcription of total adult parasite mRNA using consensus primers and ten different genes by Southern blot analysis. Further studies by Yamasaki et al. (2002) confirmed that at least three copies of cathepsin L-like proteases are found in *F. gigantica* by Southern analysis.

Early analyses of proteolytic profiles demonstrated that cathepsin L-like activity was present in *F. hepatica* ESP in all stages examined, including NEJ, 3-week-old, 5-week-old and mature flukes (Carmona et al., 1993). Gelatin-substrate polyacrylamide gel analysis showed that ESP from each of these stages contained multiple proteolytic enzymes and that the patterns of proteases obtained for NEJ ESP differed markedly from all the other stages examined (Carmona et al., 1993). The cleavage patterns of bovine serum albumin exposed to NEJ ESP were distinct from those observed with adult ESP (Wilson et al., 1998).

Early phylogenetic analysis of available cathepsin L sequences from *F. hepatica* and *F. gigantica* by Irving et al. (2003) found that *Fasciola* enzymes

were localized to a single, monophyletic clade, suggesting that the sequences arose from a common ancestral gene only after the divergence of the *Fasciola* lineage from *S. mansoni* and other species, as seen in Figure 2 below. The enzymes were divided into four clades, with the earliest duplication event separating the enzymes secreted by juvenile flukes (clade A) from those of adult enzymes. The divergence of clade B (containing FhCatL2) from clades C and D (containing FhCatL5 and FhCatL1, respectively) reflects important differences in substrate specificities between these enzyme classes: clade B (FhCatL2) enzymes can cleave substrates with proline in the P2 position and are able to cleave fibrinogen to produce a fibrin clot (Dowd et al., 1995), whereas FhCatL5 (clade C) and FhCatL1 (clade D) cannot. Amino acid 69, which lines the S2 subsite pocket in cathepsin L, has a significant effect on the ability of FhCatL2 to accommodate a P2 proline residue (Smooker et al., 2000). The cathepsins predicted to accept proline in the P2 position (clades A and B) are expected to have the least affinity for type 1 cystatins (Irving et al., 2003), cysteine protease inhibitors that mainly inhibit peptidases belonging to papain and legumain family peptidases (Turk and Bode, 1991). FhCatL1, on the other hand, cleaves substrates with hydrophobic residues in the P2 position and is specifically adapted to cleaving haemoglobin, which contains approximately 42% hydrophobic residues (Lowther et al., 2009). Irving et al. (2003) did not find segregation of cathepsin Ls from the two species of *Fasciola*, suggesting that at least some of the duplication events occurred prior to divergence of the species, approximately 19 million years ago. Recent phylogenetic analyses by Cancela et al. (2008) and Robinson et al. (2008) using an expanded repertoire of sequences have found similar clade distributions. Cancela et al. (2008), using *F. hepatica* NEJ cDNA libraries, confirmed that two cathepsin L sequences (CL3, CL4) were predominantly expressed in NEJ and their stage-specificity was confirmed by RT-PCR. CL3 was detected MALDI-TOF MS analysis of NEJ ESP proteins. In addition to these findings, Robinson et al. (2008) propose a further sub-classification of clade D (CL1), including a *F. gigantica*-specific clade (CL1C). Using tandem mass spectrometric (MS/MS) analysis of *F. hepatica* adult ESP, cathepsin L sequences

from CL1 subclade A and B were detected as well as clade CL2 (clade B) and clade CL5 (clade C). Representatives of CL1 subclade C, CL3 (NEJ-specific clade A) and CL4 (NEJ-specific clade A) were not detected in the adult ESP proteins. The clade CL1 cathepsins accounted for 67.4% of total cathepsin protein detected, CL2 accounted for 27.6% of the total secreted cathepsin protein levels, and CL5 enzymes accounted for 5.0%. Morphew et al. (2011) similarly found CL1, CL2 and CL5 sequences in adult *F. hepatica* ESP obtained *in vitro* and *in vivo*, but no matches to members of the CL3, CL4, and CL1C clades. The study also proposed an additional subclade, CL1D, and a sixth clade. A recent functional analysis of the adult-specific FhCatL5 and the NEJ-specific FhCatL1G demonstrated differences in pH stability and host substrate cleavage activity profiles (Norbury et al., 2010), reflecting the different host environments in which each of the proteases are found.

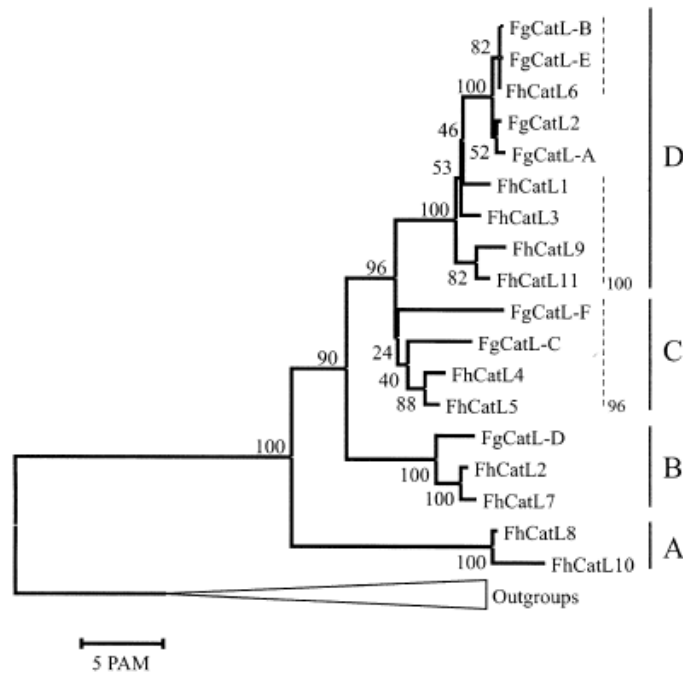


Figure 2: Bootstrap neighbour-joining phylogenetic tree of *Fasciola* cathepsin L amino acid sequences, from Irving et al. (2003).

In addition to finding a family of cathepsin L-like proteases, Heussler and Dobbelaere (1994) also found that *F. hepatica* encode cathepsin B-like proteases,

which were expressed at lower levels than cathepsin L in adult parasites. Early proteomic analyses of *F. hepatica* NEJ somatic extracts (Tkalcevic et al., 1995) and ESP (Wilson et al., 1998) found cathepsin B to be the major protease at this developmental stage. The predominant NEJ cathepsin B sequence described by Wilson et al. (1998), FhCatB1 (now known as FhCB2), had 33% and 60% identity with the cathepsin B fragments described by Heussler and Dobbelaere (1994), suggesting that the FhCatB1 from NEJ was a separate gene product from the adult cathepsin B. Meemon et al. (2004) detected abundant cathepsin B transcripts in metacercariae and NEJ in *F. gigantica*. Meemon et al. (2004) characterized the cathepsin B genes in *F. gigantica*, termed FgCatB1, FgCatB2, and FgCatB3. FgCatB1 transcripts were detected by RT-PCR in all stages, whereas FgCatB2 and FgCatB3 transcripts were expressed in metacercariae, NEJ and juvenile parasites only. FgCatB2 showed 99% identity to *F. hepatica* NEJ cathepsin B with only three amino acids different in the pre/pro region. Similarly, Cancela et al. (2008) found three cathepsin B mRNA sequences (CB1, CB2, CB3) to be expressed predominantly in NEJ (Cancela et al., 2008). CB1, CB2 and CB3 are putative orthologues of FgCatB1, FgCatB2, and FgCatB3, respectively. CB2 exhibits 99% amino acid identity to the sequence of 5-week immature *F. hepatica* cathepsin B originally reported by Wilson et al. (1998) as FhCatB1. The three cathepsin B genes were specifically detected in juvenile cDNA and not detected in adult samples and CB3 was detected in NEJ ESP by MALDI-TOF MS analysis (Cancela et al., 2008). Using a recombinant form of the FhCatB1 sequence described by Wilson et al. (1998), Law et al. (2003) found that sheep developed a humoral response within 2 to 5 weeks of infection, and rats developed a humoral response after 5 weeks of infection. A second surge in antibody response at weeks 8 to 10 post-infection was hypothesized to be the result of the expression of an adult form of cathepsin B. Meemon et al. (2004) suggest the stage-specific switching off of the FgCatB2 and FgCatB3 genes during the maturation of the parasites in *F. gigantica* implicates that these enzymes may be involved in digesting host tissues during penetration and migration to the liver, whereas FgCatB1 present in all stages may perform general digestive function. A recent

paper by Sethadavit et al. (2009) lends support to this hypothesis, as the recombinant form of FgCatB3 enzyme cleaved native fibronectin, suggesting that this enzyme may be involved in digesting host connective tissues during the fluke's penetration and migration in the host. By Western blot, cathepsin B3 was detected in the whole body extract of metacercariae and NEJ but not in 4-week-old juveniles or adult parasites.

Cathepsin B has been localized to the gut epithelia and secretory granules within the gut lumen in *F. hepatica* and *F. gigantica* NEJ (Creaney et al., 1996; Meemon et al., 2004; Sethadavit et al., 2009) and in the tissues of the reproductive system of adult *F. gigantica* (Meemon et al., 2004).

FhCatB1 (CB2) has recently been characterized by Beckham et al. (2009). FhCatB1 has an atypical preference for Ile over Leu or Arg residues at the P2 substrate position and is unable to act as an exopeptidase. FhCatB1 was active across a broad pH range (optimal activity at pH 5.5-7.0) and resistant to inhibition by cystatin family inhibitors from sheep and humans. The authors hypothesize that FhcatB1 protease functions largely as a digestive enzyme in the gut of the parasite. An inhibitor of cathepsin B1, CA-074Me, reduced the motility and viability of NEJ parasites in culture (Beckham et al., 2009).

FhCatB1 (AAD11445.1), FhCatB2 (CAD32937) and FgCatB1, FgCatB2 and FgCatB3 (Meemon et al. 2004) all have an asparagine residue 3 amino acids prior to the mature sequence and thus represent likely candidates for processing by an asparaginyl endopeptidase, similar to the processing of *S. mansoni* cathepsin B (SmCBpm1). A recombinant *S. mansoni* asparaginyl endopeptidase (SmAE) cleaved FhCatB1 to its mature 30kDa form at pH 6.5 (Beckham et al., 2006). The legumain enzymes of *F. hepatica* and *F. gigantica*, described below, may perform this cathepsin B activation function in *Fasciola spp.* NEJ.

Fasciola spp. cathepsins have been implicated as central to many pathological processes. Cathepsin L and B were recently shown to be key players in the penetration of the gut using RNAi technology to reduce cathepsin L and cathepsin B transcripts (McGonigle et al., 2008). Cathepsins are thought to be involved in the early migratory phase, creating a tract through which the parasites

migrate by cleaving collagen (Howell, 1966; Norbury et al., 2010), fibronectin and laminin (Berasain et al., 1997; Norbury et al., 2010), components of the liver stroma and capsule as well as the basement membranes. Cathepsins have also been associated with protection against host immune effector mechanisms by cleavage of immunoglobulins *in vitro* (Berasain et al., 2000; Carmona et al., 1993; Chapman and Mitchell, 1982; Norbury et al., 2010) and its association with the reduction of the number of adherent eosinophils *in vitro* (Berasain et al., 2000; Goose, 1978). Cathepsins have been found to suppress and/or modulate the T_H1 immune response and induce a non-protective T_H2 immune response, and suppress the *Bordetella pertussis*-specific IFN- γ production in mice immunised with *B. pertussis* whole cell vaccine in an IL-4 dependent manner (O'Neill et al., 2001). Cathepsin L activity suppresses the proliferation of concanavalin A (ConA)-stimulated whole blood cells by cleaving the CD4 marker on sheep and human lymphocytes (Dowd et al., 1995; Prowse et al., 2002). Dowd et al (1995; 1997) demonstrated that *F. hepatica* CL2 cleaves fibrinogen in such a way that novel interactions among subunits cause them to form insoluble clots, implicating CL2 in the prevention of excessive bleeding from severed host blood vessels.

3.1.2 Leucine aminopeptidase

Carmona et al. (1994) detected a dipeptidylpeptidase in the ESP of juvenile, immature and mature flukes that produced dipeptides which could be absorbed through the parasite digestive tract. A leucine aminopeptidase was isolated from deoxycholate soluble *F. hepatica* extracts as a 240 kDa high molecular weight complex that may represent 1-3 isoforms (Acosta et al., 1998). Piacenza et al. (1999) observed immunological responses to purified LAP in sheep experimentally infected with *F. hepatica* at weeks 10-18 post-infection. Acosta et al. (Acosta et al., 2008) isolated a full-length LAP cDNA clone from adult *F. hepatica* that had characteristic features of the M17 family of cytosolic leucine aminopeptidases, though *Fh*LAP and other flatworm orthologs constitute a well-defined cluster distant to the vertebrate enzymes. Expression of LAP was found in miracidia, metacercariae, NEJs and adult flukes by PCR of stage-specific

cDNA (Acosta et al., 2008). Using enzymatic histochemical localization (Acosta et al., 1998) and transmission immunoelectron microscopy (Acosta et al., 2008), LAP was localized in the cytosol of the intestinal epithelial cells, with a strong reactivity at the apical lamella, close to the lumen where the proteolytic events degrading host proteins takes place in adult trematoda. LAP is thought to participate in the final breakdown of small polypeptides and dipeptides previously generated by cathepsin L proteases and dipeptidase (Carmona et al., 1994), perhaps in a process similar to the later stages of haemoglobin catabolism in *Plasmodium falciparum* (Stack et al., 2007). The presence in ESP may be explained by continuous “leakages” from the apical part of gut cells as they undergo continuous remodeling between absorptive and secretory phenotypes (Robinson and Threadgold, 1975). Using immunoblotting with sera from infected patients from the Peruvian Altiplano, LAP was identified as one of two specific immunodominant antigens in adult *F. hepatica* adult ESP (Marcilla et al., 2008).

3.1.3 Legumain

Legumain-like sequences were first identified in *F. hepatica* as NEJ somatic extract N-terminal protein sequences with homology to hemoglobinase proteins (Tkalcovic et al., 1995). Two legumain sequences have been identified from adult stage cDNA in *F. gigantica*, termed Lgmn-1 and Lgmn-2 (Adisakwattana et al., 2007). The sequences are members of the asparaginyl endopeptidase family. *F. gigantica* legumain transcripts were detected in metacercariae, juveniles and adults. Immunolocalization studies found RNA and protein products in the intestinal epithelium of juveniles and adults. Legumain specific activity was only detected in the whole worm extract, not in the ESP (Adisakwattana et al., 2007). Legumain has been characterised in the trematode *Paragonimus westermani* (Choi et al., 2006) and has recently been found to be an antigenic component of the ESP of the trematode *Clonorchis sinensis* (Ju et al., 2009). This asparaginyl endopeptidase may be responsible for the activation of cathepsin B in *Fasciola spp.*, a mechanism suggested by Beckham et al. (2006).

3.2 Detoxifying molecules

3.2.1 Glutathione S-transferases

Glutathione S-transferases are a family of multi-functional enzymes involved in the cellular detoxification and excretion of many physiological and xenobiotic substances. They catalyse the nucleophilic addition of the thiol of reduced glutathione to electrophilic centres in organic compounds, rendering them more water soluble (Cervi et al., 1999). Glutathione S-transferase enzymes in *F. hepatica* were initially found to composed of multiple isozymes of approximately 25 kDa (Howell et al., 1988). N-terminal sequencing revealed that the two major components migrating at 26 kDa and 26.5 kDa contained at least three distinct sequences in the mu class of enzymes with high sequence similarity to GSTs of other helminths and mammals (Wijffels et al., 1992). Cloning of cDNAs encoding GSTs from adult *F. hepatica* by Muro et al. (1993) and Panaccio et al. (1992) revealed several different sequences, categorized by Panaccio et al. to be one of four types, represented by GST1, GST7, GST47 and GST51. The four predicted proteins share between 71 and 89% homology at the amino acid level. GST51, GST47 and GST7 are more closely related to each other (85-89%) than to GST1 (71-74%) (Panaccio et al., 1992). Creaney et al. (1995) confirmed that antibodies raised against GST51 and GST7 cross-reacted with recombinant GST7, GST47 and GST51 but not GST1. GST was immunolocalized to the parenchyma in *F. hepatica* adults and NEJ, with reactivity to GST51 also observed in the subtegumental tissues in adults. Chemale et al. (2006) carried out comprehensive proteomic analysis of the GSTs expressed by *F. hepatica* and found a new GST closely related to the sigma class in addition to the previously identified GSTs using MS/MS. A cDNA encoding a putative omega class GST was also identified (Chemale et al., 2006). A GST cDNA sequence has recently been cloned from adult *F. gigantica* (Jedeppa et al., 2009).

The expression of GST in *F. hepatica* appears to be host-dependent; GST activity in parasites from hosts relatively resistant to reinfection (rats and cattle) has been found to be lower and more restricted in range compared with flukes in

hosts susceptible to multiple infection (mice and sheep). In the case of rat flukes, there was little variation in isozyme profiles, whereas cattle flukes exhibited more variation than sheep flukes (Miller et al., 1993).

3.2.2 Cu/Zn SOD

Superoxide dismutase (SOD) (EC 1.15.1.1), the main superoxide (O_2^-) radical scavenger, protects cells from oxidant-mediated damage. This enzyme catalyses the dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Piacenza et al., 1998). Piacenza et al. (1998) found SOD activity in *F. hepatica* ESP, with high levels in ESP from 3-week-old and 5-week-old immature migrating flukes and moderate amounts in the mature parasite ESP, suggesting a progressive decrease in specific activity as the parasite matures. Differences in SOD isoenzyme profiles between the extracts were observed in native polyacrylamide gel electrophoresis: the somatic and detergent-soluble extracts exhibited one band of activity while the ESP from immature and adult flukes contained two and three migrating bands, respectively. Kim et al. (2000) purified, cloned, and characterised a Cu/Zn-SOD from *F. hepatica*. The enzyme consisted of two identical subunits, each with an apparent molecular mass of 17.5 kDa and showed antigenicity against sera from affected humans and cattle.

3.2.3 Thioredoxin-associated proteins

F. hepatica is exposed to reactive oxygen species such as O_2^- , H_2O_2 and the hydroxyl radical (OH \cdot). The first line of defence involves removal of O_2^- by SOD, producing O_2 and H_2O_2 . The second involves removal of H_2O_2 , which in most cells is normally achieved by catalase and various peroxidases. However, *F. hepatica* does not contain catalase for detoxification of H_2O_2 . In the filarial nematode parasites, metabolism of H_2O_2 is achieved via peroxiredoxin and thioredoxin activity (Selkirk et al., 1998). Thioredoxin-associated proteins include peroxiredoxin, thioredoxin and thioredoxin reductase.

Peroxiredoxin, or thioredoxin peroxidase, was the first member of the thioredoxin family to be isolated from *F. hepatica* (McGonigle et al., 1997). A cDNA was isolated from a *F. hepatica* expression library using antiserum

prepared against a high molecular mass fraction of *F. hepatica* adult ESP (Dalton et al., 1996). Thioredoxin peroxidase may play a role in protecting the fluke against intracellularly produced reactive oxygen species (ROS), thus acting as a general reductant within the cell, or may function as a defence against external oxidants generated by the host immune response (McGonigle et al., 1997). Thioredoxin peroxidase induces the recruitment of alternatively activated macrophages to the peritoneum in mice (Donnelly et al., 2005).

Thioredoxin, the next component of the thioredoxin-associated protein chain, was isolated from *F. hepatica* adult cDNA. The recombinant protein was found to be biologically active and activated thioredoxin peroxidase from *F. hepatica* (Salazar-Calderon et al., 2001). The thioredoxin protein was detected in adult *F. hepatica* ESP using antiserum against the recombinant GST-thioredoxin at approximately 12kDa. *F. hepatica* thioredoxin appears to be only weakly antigenic for bovine T cells (Shoda et al., 1999).

Thioredoxin reductase (TrxR) is a selenocysteine flavin adenine dinucleotide (FAD)-containing enzyme with some structural and functional similarities to glutathione reductase. The TrxR mechanism involves the transfer of reducing equivalents from NADPH to a disulfide bond in the enzyme by way of FAD. TrxR was isolated from *F. hepatica* adult deoxycholate-soluble extract and immunolocalized in cells located within the parenchyma and in the testes, but it was not found in the tegument of adults (Maggioli et al., 2004). Immunoblotting revealed reactive bands at 55 and 60kDa.

Thioredoxin-associated proteins have not been described in *F. gigantica* to date.

3.2.4 Protein disulphide isomerase

A cDNA clone encoding a putative protein disulphide isomerase (PDI) enzyme was isolated from an adult *F. hepatica* cDNA library by immunological screening using a rabbit antiserum against ESP. PDI is a member of the thioredoxin superfamily. A protein of 55 kDa was detected in adult *F. hepatica*

ESP using anti-recombinant PDI sera. This protein is hypothesized to function as an antioxidant protein (Salazar-Calderon et al., 2003).

3.3 Other proteins of interest

3.3.1 FABP

FABP was identified as a component of an antigen complex from a crude somatic extract of *F. hepatica* that protected mice against infection (Hillyer and Cervoni, 1978; Rasmussen et al., 1985). This fraction showed up to 78% protection in the mouse (Hillyer, 1985), and 55% protection in cattle (Hillyer et al., 1987). Hillyer et al. (1988) identified the main protective antigen in the complex as a 12 kDa protein, named Fh12. A cDNA encoding a 15 kDa was isolated by immunological screening using anti-Fh12 rabbit serum and found to have significant homology to a 14 kDa *S. mansoni* FABP (Rodriguez-Perez et al., 1992). Trematodes cannot synthesise lipids, such as phospholipids, triacylglycerol, and cholesterol, *de novo* and these must be made from building blocks obtained from the host (Meyer et al., 1970). nFh12 and rFh15 appear slightly different, as nFh12 is reactive with sera from mice with 2-week *F. hepatica* infection while rFh15 is reactive with sera from mice after the sixth week of *F. hepatica* infection (Hillyer, 1995), suggesting that rFh15 could be one of the proteins of the nFh12 complex and possibly one of the less immunogenic molecules. Bozas and Spithill (1996) found two additional FABPs in *F. hepatica* somatic extracts. Espino et al. (2001) demonstrated that nFh12 is a complex of at least eight isoforms with identical molecular mass but different isoelectric points. The study found that in infected rabbits, antibodies to nFh12 appear by the second week postinfection, whereas antibodies to rFh15 appear much later, at 6 weeks postinfection. Four acidic forms (Fh12₁₋₄) showed more immunological identity with rFh15 than with nFh12, and were poorly reactive with sera from rabbits at weeks 2-4 post-infection. The two acidic forms, denoted Fh12₅ and Fh12₆, and the neutral/basic forms, denoted Fh12₇ and Fh12₈, showed more immunological identity with the native nFh12 molecule than with the recombinant rFh15. These results suggest that rFh15 could be one of the acidic forms of nFh12 and that it

may be one of the less immunogenic or immunoprotective members of the nFh12 protein complex (Espino et al., 2001).

A cDNA from an adult *F. gigantica* library was cloned using primers specific for the *F. hepatica* FABP sequence (Smooker et al., 1997). *F. gigantica* FABP1c and *F. hepatica* FhFABP1 (Rodriguez-Perez et al., 1992) are highly conserved, exhibiting 94% nucleotide identity and 89% amino acid identity, or 94% similarity if conservative substitutions are taken into account. There is only 72% sequence identity with the second known *F. hepatica* FhFABP2. A second *F. gigantica* FABP sequence was cloned by Sirisriro et al. (2002), and immunolocalized at high abundance in parenchymal cells and reproductive tissues and in low concentration in the tegument and caecal epithelium.

3.3.2 Enolase

Enolase was detected in *F. hepatica* adult ESP at 47 kDa by immunoblotting using sera from human fasciolosis patients (Bernal et al., 2004). The *F. hepatica* mRNA encoding enolase was previously cloned in a study aimed at identifying mRNA regions involved in trans-splicing (Davis et al., 1994). Bernal et al. (2004) determined that *F. hepatica* enolase is a human plasminogen-binding protein. Enolase has been identified in the ESP of adult *F. hepatica* (Jefferies et al., 2001; Morphew et al., 2007; Robinson et al., 2009) and has also been found to be an antigen specifically recognized by patients in the Peruvian Altiplano by immunoblotting (Marcilla et al., 2008).

3.3.3 Saposin

Reed et al. (1998) identified a sequence that was expressed at levels at least ten-fold higher in immature parasites relative to adults. The encoded protein, termed *FhSAP-1*, is a novel member of the natural killer (NK)-lysin or saposin-like protein family of structurally related proteins that is most closely related to the amoebapore polypeptides of pathogenic *Entamoeba histolytica* (Reed et al., 2000). A second cDNA encoding a putative saposin molecule, termed *FhSAP-2*, was isolated and cloned (Espino and Hillyer, 2003). *FhSAP-2* was highly reactive with sera from rabbits infected with *F. hepatica* at weeks 2-14 post-infection as

well as with sera from humans with chronic fasciolosis. Antisera against recombinant *FhSAP-2* reacted with adult *F. hepatica* ESP by Western blot, revealing major bands at 11.5 kDa and 2 minor bands at 46 kDa and 67 kDa. *rFhSAP-2* also exhibits a strong lytic activity on human erythrocytes and peripheral blood mononuclear cells (Espino and Hillyer, 2003).

Grams et al. (2006) identified saposin in an immunoscreen of adult-stage *F. gigantica* cDNA library using antibodies against ESP. In immature parasites, SAP-1 RNA is the predominant product: in adults, SAP-2 and SAP-3 are more abundant. Transcripts of the three saposins are present from the metacercarial stage to the adult stage and are located in the gut epithelium.

3.3.4 Haemoglobin

A Hb-like protein was isolated from *F. hepatica* adult ESP with an apparent molecular mass of over 200 kDa. N-terminal amino sequence analysis revealed no similarity between the *F. hepatica* haemoglobin and other vertebrate or invertebrate haemoglobins, but this protein contains a haem group and absorption spectra characteristics that are similar to haemoglobins. Antibodies to the haemoglobin molecule can be detected in the sera of *F. hepatica*-infected bovines as early as one week after infection (McGonigle and Dalton, 1995). This protein, when used as a vaccine in combination with CL1 or CL2, shows moderate protection and significant anti-embryonation effects against *F. hepatica* eggs (Dalton et al., 1996).

A recent study by Dewilde et al. (2008) found that *F. hepatica* expresses two isoforms of haemoglobin that differ at two amino acid sites and are approximately 17 kDa. This study showed that, despite the potential immunogenic character of the fluke haemoglobin, vaccination of calves with recombinant *F. hepatica* HbF2 did not lead to protect against infection (Dewilde et al., 2008). The protein obtained by Dewilde et al. (2008) differs both in protein sequence and in molecular weight from the Hb identified by McGonigle and Dalton (1995).

3.3.5 Paramyosin

Cancela et al. (2004) isolated paramyosin, a myofibrillar protein, from adult *F. hepatica*. The protein migrated at 97 kDa under reducing conditions, and 200 kDa under non-reducing conditions. Immunohistochemical studies localised paramyosin within the musculature of *F. hepatica*, the surface lamellae of gut and the testes of adult parasites and within the tegumental surfaces of the adult parasite. Estuningsih et al. (1997) isolated a 94 kDa paramyosin from adult *F. gigantica* extract, which cross-reacted with antisera raised to paramyosin of *F. hepatica*. Though vaccination using paramyosin induced very high antibody titres in cattle, this was not protective against *F. gigantica* challenge infection (Estuningsih et al., 1997).

3.3.6 Protease inhibitors

The first description of a protease inhibitor expressed by *F. hepatica* was reported by Bozas et al. (1995). Several polymorphisms of the 6.7 kDa Kunitz-type inhibitor were observed, suggesting more than one isoform. The protein localized to the gut, parenchymal tissue and the tegument of adult *F. hepatica*.

A putative cystatin was identified in *F. hepatica* from mRNA fragments specifically or over-expressed in NEJ (Khaznadji et al., 2005). The sequence encoded a multi-domain cystatin with homology to cystatin family 2. Semi-quantification of RNA levels by PCR showed that while the transcript is detectable after 20 amplification cycles in the NEJ sample, it was only detectable in adults after 25 cycles. This cystatin was found to be a potent inhibitor of papain and the major cysteine protease of *F. hepatica*, cathepsin L1. The *F. hepatica* cystatin was hypothesized by the authors to regulate cysteine protease activity in the NEJ stage of the parasite or to contribute to immunomodulation of host.

Tarasuk et al. (2009) has recently found a type 1 cystatin (Stefin) as a major component of *F. gigantica* ESP. FgStefin-1 transcripts were found in metacercariae, juvenile and adult stages and two epitope-sharing cystatin isoforms were detected by 2-dimensional immunoblotting. FgStefin-1 is partially complexed with cathepsin L in ESP, and is released in approximate molar ratios

of 1:1 in ESP. This FgStefin-1 is abundant in the tegument and the intestinal epithelium. Sera of an experimentally infected rabbit reacted with recombinant FgStefin-1 starting at 8 weeks postinfection. FgStefin-1 showed nanomolar inhibition constants for mammalian cathepsin B, L and S and secreted cysteine proteases of the parasite. The results suggest a protective function of FgStefin-1, regulating intracellular cysteine protease activity, and possibly protection against extracellular proteolytic damage to the parasite's intestinal and tegumental surface proteins. The protein may also have immunomodulatory roles, such as observed in *Brugia malayi* microfilarial serpin that specifically inhibits proinflammatory serine proteases from human neutrophils (Maizels et al., 2001; Zang et al., 1999).

3.3.7 Proteomic profiles of *F. hepatica* ES

Jefferies et al. (2001) performed the first comprehensive MS analysis of *F. hepatica* ESP collected *in vitro*. This group identified 29 of the 60 prominent proteins separated by 2-dimensional electrophoresis (2DE) and identified the proteins by peptide mass fingerprinting (PMF). Of the proteins found, three were FABPs (A44864: FABP homolog Fh15, A44638: FABP type 1, CAB65015: FABP), five were detoxifying enzymes (AAD30361: *F. hepatica* Cu/Zn SOD, P09670: *Ovis aries* Cu/Zn SOD, CAA06158: Thiol-specific antioxidant protein, P91883, Thioredoxin peroxidase, GST), three were cathepsin L proteases (AAB41670: Secreted cathepsin L 1, CAC12806: cathepsin L, T09259: cathepsin L-like proteinase) with hits to many other cathepsin L sequences, and one protein involved in intracellular metabolic processes (A53665: Enolase).

Morphew et al. (2007) expanded on the work carried out in Jefferies et al. (2001) by analyzing ESP released both *in vitro* and *in vivo* by in host bile *F. hepatica* parasites in cattle using 2DE and PMF. Most cathepsin L sequences (CAA80446, AAC47721, AAF76330, AAK38169, AAF44678) were detected both *in vitro* and *in vivo*, with one sequence (AAM11647) detected *in vitro* only. Certain proteins were identified as host proteins only *in vivo*, including Cu/Zn SOD (P09670), carbonic anhydrase II (P00922), regucalcin (NP_776382), enolase 1 (NP_776474), serum albumin precursor (P14639) and transferrin (NP_803450).

Additional proteins were detected *in vitro* only, including FABP type II (Q7M4G1), glutathione *s*-transferase 26kDa 51 (GST51) (AAB28746), glyceraldehyde phosphate dehydrogenase (AAG23287), actin (AAA826603) and FABP type III (CAB65015). This study was limited by the number of sequences available in both *Fasciola spp.* database as well as the cattle genome, which has only recently been published (Elsik et al., 2009).

Robinson et al. (2008b) identified the cathepsin L sequences released by *F. hepatica* adult parasites *in vitro*, and found that they are principally FhCL1A (AF490984, AY519971, AY519972, AY573569), FhCL1B (AJ279092) and FhCL2 (Z22765) clade enzymes, with a smaller proportion of FhCL5 (AF271385) clade enzymes.

Most recently, Robinson et al. (2009) published a comprehensive study that included a predictive analysis of secretory proteins of over 14 000 adult *F. hepatica* ESTs using the EST2Secretome pipeline. The group predicted 160 cDNAs encoding secreted proteins, 41% of which encoded cathepsin L cysteine proteases. The study also included MS/MS analysis of ESP proteins released *in vitro* by adult *F. hepatica* parasites. Of the 22 proteins identified in adult *F. hepatica* ESP, 13 corresponded to cathepsin L sequences from the FhCL1, FhCL2 and FhCL5 clades. Three putative sequences predicted to be secreted by the EST2Secretome pipeline were also found, including saposin-like protein 3, a peptidyl-prolyl cis-trans isomerase and a protein with homology to an uncharacterized *C. sinensis* secretory protein. In addition to these proteins, four FABP (FABP1, FABP2, FABP3 and Fh15), two redox enzymes (peroxiredoxin and thioredoxin) and a putative novel prolylcarboxypeptidase were identified by MS but were not predicted to be secreted. Proteins predicted to be secreted by this pipeline, but not detected by proteomic analysis, include novel cathepsin B sequences (designated B4-B10 in the study), legumain, serine carboxypeptidase, two trypsin-like enzymes, and egg-shell vitelline protein B1.

In addition to the MS/MS analysis of *F. hepatica* adult ESP, Robinson et al. (2009) also carried out an MS/MS analysis of *F. hepatica* NEJ ESP proteins. The study found 29 different proteins, including the following proteases:

7 cathepsin L3 sequences, one cathepsin B, and two asparaginyl endopeptidase-like proteases. Other protein matches included enolase, three fatty acid binding proteins (Fh2, Fh3 and FH 15) and peroxiredoxin. A further 16 different putative peptides were identified following searches of the *F. hepatica* EST database, including the metabolic enzymes fructose-bisphosphate dehydrogenase, malate dehydrogenase and an ATPase. Other matches included putative structural proteins such as calponin, spermadhesin and histones (H2A, H3B, and H4), redox enzymes (thioredoxin and peptidyl-prolyl cis-trans isomerase) and an uncharacterized protein with predicted transmembrane regions. Finally, molecules with roles in protein turnover such as ubiquitin and a putative serpin were also identified.

Robinson et al. (2009) also carried out an MS/MS analysis of *F. hepatica* immature ESP. They found a total of 45 different proteins. Of these, 34 were matched to previously identified *Fasciola* cDNAs and 11 corresponded to putative proteins encoded by novel ESTs. Mass spectrometry data also matched to peptides encoded by a further 2 *F. hepatica* EST sequences that lacked conserved protein domains and could not be assigned putative functions based on BLAST searches. Of the 45 positively-matched proteins, 22 were proteases and included 14 cathepsin Ls, four cathepsin Bs, three asparaginyl endopeptidases (legumains) and a newly discovered prolylcarboxypeptidase. The remaining 23 proteins secreted by the immature liver stage parasites included glutathione S-transferase (GST) sigma class enzyme and four isoforms of mu class GSTs (GST1, GST7, GST47 and GST51), four fatty acid-binding proteins (FABP1, FABP2, FABP3, and Fh15), two saposin-like proteins (SAP1 and SAP3), two enzymes of glycolysis (enolase and triosephosphate isomerase) and two enzymes involved in cell redox homeostasis (peroxiredoxin and protein disulphide isomerase). Other significant peptides matched included annexin, ferritin, ubiquitin, a 14-3-3 protein, a multi-cystatin and a putative ABC transporter protein.

The detection of cytosolic proteins by MS/MS analysis by Robinson et al. (2009), including fructose-bisphosphate dehydrogenase, malate dehydrogenase, enolase, triosephosphate isomerase, histone proteins, and ubiquitin suggest that

NEJ were damaged or dying during the ESP collection process. The results may therefore be taken as a preliminary study of the proteins detected in the sample and should be verified by additional experimentation before concluding that they are secreted by the parasites.

To date, no equivalent MS analyses have been reported on *F. gigantica* ESP.

3.4 Antigenic profiling of *F. hepatica* and *F. gigantica* ESP

F. hepatica and *F. gigantica* infected sheep and calves develop clear and early total immunoglobulin and IgG₁ responses to *F. hepatica*- and *F. gigantica*-ES products (FhES, FgES) (Phiri et al., 2006). This predominant IgG₁ response to *F. hepatica* in cattle has been found in both chronically infected and previously naïve animals (Clery et al., 1996; Hoyle et al., 2003) as well as single-dose and trickle *F. hepatica* infections (Bossart et al., 2000a). There is also a strong but late IgG₂ response to FhES in *F. hepatica*-infected cattle at week 9 post-infection, and an IgA response at week 17 post-infection (Phiri et al., 2006). Sheep, on the other hand, do not have a strong IgG₂ response to *Fasciola spp.* ESP and have biphasic IgA and IgE responses in *F. hepatica* and *F. gigantica* infections (Hansen et al., 1999). Mulcahy et al. (1998) found that an elevation of IgG₂ was associated with protection in their *F. hepatica* cattle immunization studies.

Zhang et al. (2004a) profiled the antigens in FhES and FgES recognized by sheep infected with *F. hepatica* and *F. gigantica*. Using homologous infection sera, 25 bands were observed in FhES ranging from 13.4 to 135.1 kDa, with three clusters of intense bands (52.3 and 55.3 kDa; 26.1, 27.3 and 28.5 kDa; 12.9, 13.6 and 15.1 kDa), whereas 31 bands were observed in FgESP ranging from 12.0 to 127.5 kDa, with two clusters of intense bands (27.1 through 31.9 kDa; 16.1 kDa). Previous studies carried out with FhES found that *F. hepatica*-infected sheep sera recognized major antigens at 200, 97.4, 69, 46, 30, 21.5, and 14.3 kDa (Chauvin et al., 1995) and that cattle infected with *F. hepatica* recognized antigens at 82-96, 68-70, 55, and 52 kDa (Hoyle et al., 2003) at 4-5 weeks post-infection. Zhang et al. (2004a) found that most of the major antigens in FhES and FgES were

commonly recognized by sera from sheep infected with either *F. gigantica* and *F. hepatica*. However, *F. hepatica* infected sheep sera reacted more strongly with most of the lower molecular mass antigens (from 9.0 to 31.9 kDa in FhESP, from 11.5 to 36.6 kDa in FgESP) than *F. gigantica* infected sheep sera. *F. gigantica* infected sheep sera primarily recognized the higher molecular mass antigens (from 21.6 to 40.2 kDa in FhESP, from 7.8 to 71.2 kDa in FgESP). Chauvin et al. (1995) and Hoyle et al. (2003) also reported mass-dependent differences in antigen recognition; they found a sequential recognition of high molecular weight (>50 kDa) followed by low molecular weight proteins (28-30 kDa) during the course of *F. hepatica* infections. Hoyle et al. (2003) found that the immune response against higher molecular mass proteins (>50 kDa) was associated with a significant resistance to reinfection in cattle.

Many studies reported the main *F. hepatica* ESP antigen in sheep and cattle infections to be in the 26-31 kDa range (Bossaert et al., 2000a; Santiago and Hillyer, 1988; Sexton et al., 1991), corresponding to the molecular mass of cathepsin L.

Antigenic studies suggest that early recognition of adult ESP in *F. hepatica* and *F. gigantica* infections is due to similarities between the antigens of the immature and mature forms of the parasite (Sexton et al., 1991; Phiri et al., 2006) and that juvenile flukes secrete enough antigenic ESP to provoke a detectable antibody response (Phiri et al., 2006).

4. Proteomics

Biomarkers are proteins that are differentially expressed in pathological states when compared to healthy controls. They can be early indicators of disease or indicators of disease progression and treatment response; they can also represent novel targets for drug development. The approaches used in proteomic profiling have been largely tied to technological advances; therefore a broad overview of the techniques used in profiling will be presented, followed by the specific challenges that are faced when working with serum and plasma in biomarker discovery.

4.1 Technological framework of proteomics

4.1.1 Early protein profiling and 2-dimensional electrophoresis (2-DE)

Early proteomic profiling was based on the comparison of migration patterns using electrophoresis in the moving boundary system (Tiselius, 1937) and within a polyacrylamide matrix (PAGE) (Raymond and Weintraub, 1959), which separated proteins according to mass. The main drawback to this approach is the inability to resolve proteins of identical masses in a complex sample. Electrophoresis of proteins was adapted to separate them by charge by Svensson in 1962 using a stabilized gradient, a process known as isoelectric focusing (IEF). This approach cannot separate proteins with similar isoelectric points (pI). By combining both IEF as an initial separation technique, followed by SDS-PAGE in a second dimension, Kenrick and Margolis (1970) introduced a technique that separated proteins by both their pI and mass, making it possible to separate thousands of *Escherichia coli* proteins on a single 2-D gel (O'Farrell, 1975). This process is commonly referred to as two-dimensional gel electrophoresis, or 2-DE. Each of the spots identified by 2-DE could be subjected to Edman degradation (Edman and Begg, 1967) to determine its N-terminal sequence, but this technique required hundreds of picomoles of material. Spots could also be identified by co-migration with known proteins. 2-DE is a powerful technique for the separation of proteins, but the technique at the time required careful preparation of carrier ampholyte pH gradients in capillary tubes, limiting loading capacity and reducing reproducibility. Advancements in RNA and DNA sequencing and amplification by PCR (Saiki et al., 1985) lead to a shift from predominantly proteomic-based approaches to expression profiling to include genomic-based approaches. Expression profiles were measured by SDS-PAGE, Northern blotting and PCR (Penque, 2009).

4.1.2 2-DE and MS

There was a reemergence of the use of 2-DE in the mid-1990s as a result of two important developments in technology: immobilized pH gradients (IPG) which increased the 2-DE reproducibility (Bjellqvist et al., 1982), and advances in

MS technology that extended its use to include biological molecules, including peptides. Previous MS analyses were limited to molecules <1 000 Da, but novel technologies extended the range of analysis. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization MS (MALDI MS) are commonly used to volatilize and ionize peptides and proteins for mass spectrometric analysis (Aebersold and Mann, 2003). ESI ionizes analytes out of solution and therefore can be coupled with an upstream separation of peptides by liquid-based separation methods, such as chromatographic or electrophoretic techniques. In MALDI MS, samples are combined with a matrix that facilitates ionization and desorption of peptides spotted onto an inert surface. The proteins are ionized by a laser pulse and the ions are directed towards the MS detector. MALDI MS is normally used to analyze relatively simple peptide mixtures, whereas liquid chromatography ESI-MS systems (LC-MS) are used for analysis of complex samples (Aebersold and Mann, 2003). MS instruments contain analyzers that can either measure the mass of the ion (time-of-flight (TOF), quadrupole) or can trap the ions prior to MS analysis (Fourier transform cyclotron (FT-MS), ion trap). Tandem MS instruments (MS/MS) include two analyzers, allowing for the selection of ions, fragmentation, and measurement of their M/z ratio.

Previous approaches to proteomic analysis included the digestion of proteins using endoproteases, such as trypsin, to generate a fingerprint of the protein. The peptides generated through this proteolytic process are not only easier to elute from the gel matrix than whole proteins, but the peptide mass fingerprint (PMF) that is generated from MS analysis of the peptide can be compared to the databases of calculated fragments of digested proteins for identification (Henzel et al., 1993). In MS/MS, the sequence of the peptide is not determined *de novo*, but rather is determined by comparing the spectra with theoretical peptides. The PMF or peptide sequences are used to identify the protein of interest by searching databases using algorithms such as SEQUEST or MASCOT (Aebersold and Mann, 2003; Canas et al., 2006; Pandey and Mann, 2000).

The analysis of data collected by MS is largely dependent on the availability of genomic information and the ability to manipulate and integrate the information using bioinformatics. MS analysis is therefore largely restricted to species with comprehensive sequence databases available. Large-scale proteomic analyses are therefore commonly collaborative projects incorporating all of these important aspects. The Plasma Proteome Project that is organized by the Human Proteome Organization (HUPO) is an example of an integrated approach to understanding the human plasma proteome (Omenn, 2004). This project represents a unified effort to collect data about the proteome of plasma, but has also been a key player in the standardization of specimen collection and analysis to ensure that data from around the world can be properly compared.

In order to accurately assess biomarkers using 2DE, the dye used for visualization must be sensitive and provide accurate densitometry analysis. Colorimetric methods have been used, including colloidal Coomassie brilliant blue (CBB)-based stains, with a staining sensitivity limit of 8-100 ng/spot (Candiano et al., 2004), silver staining, with <1-10 ng/spot and fluorescent dyes, with sensitivities of 1-2 ng/spot. CBB-based dyes are not sufficiently sensitive for the detection of low-abundance proteins in the presence of high-abundance proteins; silver staining is not as reproducible as CBB or fluorescent staining and is not MS-compatible. Fluorescent stains have become increasingly popular due to their comparatively wide linear dynamic range ($>10^3$) and their sensitivity (Penque, 2009). In differential in-gel digestion (DIGE), different cyanine dyes (e.g. CyDye2, CyDye3, CyDye5) are covalently linked to healthy, diseased, and internal control samples and equal amounts of each of the samples are mixed and separated by 2-DE, allowing direct densitometry comparison of samples within a gel and minimizing the gel-to-gel variation inherent in 2DE analysis. The internal control serves as an internal standard, enabling both inter- and intragel matching. DIGE is a sensitive technique, capable of detecting 0.5 fmol of protein, and this detection system is linear over a 410 000-fold concentration range (Minden, 2007). In order to accurately identify biomarkers of infection, spots on 2DE gels must be identified, matched between gels, the density must be normalized and

quantified (Penque, 2009). Given the advantages of DIGE analysis in minimizing the variation in each of these requirements for image analysis, DIGE has become the recommended 2DE method in proteomics (Gorg et al., 2004).

4.1.3 SELDI-TOF MS

Surface-enhanced laser desorption ionization time-of-flight MS (SELDI-TOF MS) is used to profile proteins in biological samples, generating a proteomic fingerprint for a particular sample that can be used in diagnostics. The system is composed of three major components: the protein chip, mass analyzer, and data analysis software (Issaq et al. 2007). Profiling begins with the binding of sample to chemically active protein chip surfaces using traditional chromatographic approaches, including hydrophobic, ion-exchange, and metal binding. The sample is coated with matrix, similarly to MALDI-TOF MS, and is analyzed by MS. The result is a mass spectrum of M/z values and intensities of the bound proteins/peptides. The basic principle is very similar to MALDI-TOF MS, but the outputs of SELDI-TOF MS include a measure of intensity for each peak that can be compared between spectra using dedicated software. The analysis software can recognize peaks in the spectra, compare two or three groups of spectra, and conduct statistical analyses to identify significant proteins differences between samples (Issaq et al. 2007). The advantage of this method over other MS-based methods is the throughput and the ability to compare the relative abundance of particular peaks statistically.

The first study published using SELDI-TOF MS technology reported that the serum protein profile could be used to identify individuals with ovarian cancer. Serum samples from 50 healthy women and 50 women in different stages of ovarian cancer were analyzed. These initial samples were used as a training set to identify markers that discriminated cancer from noncancer cases. The discriminatory proteomic pattern was then tested on a set of 116 masked serum samples and was found to have 100% sensitivity and 95% specificity in the identification of blinded ovarian cases and nonmalignant cases (Petricoin et al., 2002b). To achieve higher specificity, the group used a hybrid quadrupole TOF-

MS to obtain higher-resolution proteomic profiles and were able to correctly identify 43 samples as healthy, 68 as ovarian cancer, including 18 stage I patients with 100% sensitivity and specificity in the validation test (Conrads et al., 2004). These studies generated an increase in interest in this technology, and analyses initially were applied to the early diagnostic or prognostic biomarkers of cancer, such as prostate cancer (Lehrer et al., 2003; Petricoin et al., 2002a; Semmes et al., 2005b), ovarian cancer (Kozak et al., 2003; Zhang et al., 2004b), pancreatic cancer (Koopmann et al., 2004; Rosty et al., 2002) and renal cancer (reviewed in Tolson et al., 2004; Won et al., 2003; Xiao et al., 2005) . The technique was also applied to the study of serum biomarkers of infectious diseases such as Severe Acute respiratory Syndrome (Poon et al., 2004; Yip et al., 2005a), African Trypanosomiasis (Papadopoulos et al., 2004) and Chagas' disease (Ndao et al., 2010b).

The main areas that are problematic with the SELDI-TOF MS technology include sample preparation, generation of reproducible data and identification of signature traces. In an early review of SELDI-TOF MS technology, Diamandis (2004) looked into early studies of cancer biomarkers, particular studies into prostate cancer and ovarian cancer. The author's analysis of five papers of prostate cancer and two studies of ovarian cancer found that the distinguishing peaks between cancer and non-cancer patients are very different between the various research groups, and that well-established cancer biomarkers were not identified. Baggerly et al. (2004) also reviewed the early datasets published by Petricoin et al. (2002) and found that the results were not reproducible across experiments. The results could not be reproduced in this study due to baseline corrections in two of the three experiments. In one experiment, there was evidence of a major shift in protocol mid-experiment that could bias the results of the study. In another dataset, structure in the noise regions of the spectra allowed the researchers to distinguish normal from cancer, suggesting that the normals and cancers were processed differently. It is therefore critical to use standard operating procedures when using this technology (Ndao et al., 2010a). As for protein identification, without coupling the SELDI interface to a tandem MS

instrument, it is difficult to identify the peaks directly using this method. This has been a significant hurdle in translating a proteomic pattern into an antibody- or DNA-based assay. While few studies actually proceed to identify peaks of interest, those that do typically find that they are intact, or fragments of, highly abundant proteins. These proteins are generally acute-phase or inflammatory response proteins and are unlikely to have the necessary specificity to diagnose a particular cancer (Issaq et al., 2007).

4.1.4 Multidimensional protein identification technology (MudPIT)

Washburn et al. (2001) identified 1,484 proteins in a large-scale analysis of *Saccharomyces cerevisiae* proteome using multidimensional protein identification technology (MudPIT). The technique couples successive separation of proteins by liquid chromatography, first with a reverse phase column followed by a strong cation exchange column, and the HPLC is directly coupled to an ESI-MS. The group reported that the technique permitted the same sensitivity of identification of low-abundance proteins, proteins with extremes in pI and mass and integral membrane proteins as expected with other techniques.

MudPIT can be used to measure quantitative differences between samples by differentially labelling protein samples using the following techniques: culturing cells in media that are isotopically enriched or isotopically depleted, tagging at specific sites using isotope-coded affinity tags (ICAT) or through incorporation of ^{18}O water during proteolysis. The labelled samples are analysed simultaneously by MS or MS/MS. The pattern of isotopic differences in the mass spectra can be used to determine the relative abundance of proteins at specific M/z (Aebersold and Mann, 2003).

The disadvantage of using differential labelling in MS for the relative quantitation of peptides and proteins are the cost and time associated with the additional preparatory steps and analysis of spectra as well as the limitation of sample size. There is evidence to support the relationship between the level of sampling observed for a protein and the relative abundance of the protein in the mixture in comparison of MS or MS/MS output. Liu et al. (2004) demonstrated a

linear relationship between the number of spectra attributed to a protein (spectrum counting) and relative abundance in a range of two orders of magnitude. Zybailov et al. (2005) reported that spectrum counting and comparison of peak areas of individual peptides in spectra were strongly correlated when used to determine quantitative changes in protein expression, and that spectral counting was more reproducible over a wider dynamic range. Due to its effectiveness within two orders of magnitude, this approach requires the depletion of high abundance proteins, but otherwise requires minimal sample handling when compared to ICAT experiments, for example. This technique measures differences that are more than 3-fold and is not ideally suited to the relative quantification of low abundance proteins that are identified using one or two peptides. Qian et al. (2005) used both approaches in the identification of acute phase reactants resulting from administration of lipopolysaccharide.

4.1.5 Immunoprecipitation methods

When studying biomarkers, the use of immunoproteomics may be particularly helpful in identifying key players in the host-parasite interaction. The use of 2DE and MS is known as serological proteome analysis (SERPA) (Penque, 2009). This approach is particularly useful when working with cell lysates and can be used to compare the immunoreactivity of infected sera to normal sera. Typically the number of spots is increased in diseased patients and is used to identify spots for identification from preparative 2-DE. This approach has been used in autoimmune diseases (Selmi, 2007), in cancer (Klade et al., 2001) and to identify antigens from infectious organisms such as *Helicobacter pylori*, *Chlamydia trachomatis*, and *Borrelia garinii* (Klade, 2002; Nilsson, 2002). This approach promises to be an important tool in the identification of vaccine candidates.

Co-immunoprecipitation (CoIP) is a related technique that is based on the use of antibodies to a given protein to be fixed onto a protein A or G gel support. A sample is then run through the column under mild conditions and the ligand for the antibody is bound along with any proteins that may interact with the targeted

protein. The coprecipitated proteins are then eluted from the column and can be identified by MS or 2-DE and MS. This technique can therefore be used as a preparative step as well as a determine the ‘interactome’ of a given protein (Penque, 2009).

4.1.6 Other approaches

Proteomic approaches have also included studies of post-translational modifications (PTM) of proteins. Differences in post-translational modifications can affect protein folding, stability, cellular localization, recognition and immune reactions. Certain studies have therefore focused on differences in glycosylation and phosphorylation between control and diseased states. Differences in phosphorylation can be determined by incorporating $^{32}\text{P}/^{33}\text{P}$ orthophosphate or ^{14}C -labeled sugar into cell culture lines, using PTM-specific fluorescent stains, immunoblotting using specific antibodies or lectins with reporting groups such as peroxidase or phosphatase in a technique similar to Western blotting (Penque, 2009). Post-translational modifications can also be detected by determining the mass of digested fragments by MS or MS/MS (Aebersold and Mann, 2003).

4.1.7 Pitfalls of proteomic analysis

An important limitation of LC-MS/MS is the interpretation of the MS/MS data to derive a list of identified peptides and their corresponding proteins. This task involves distinguishing correct peptide assignments from false identifications among database search results and relies heavily on the researcher’s expertise in spectral interpretation of the database peptide assignments. Alternatively, filtering criteria based on database search scores can be used (Aebersold and Mann, 2003). The criteria for the correct identification of a protein are therefore evolving.

5. Working with serum and plasma: challenges and solutions

5.1 Search for biomarkers in serum and plasma

Plasma and serum proteins are used in biomarker studies because the configuration of circulating serum proteins is considered to reflect the status of

tissues in the patient. The proteins present in plasma and serum do not have a corresponding genome and transcriptome, therefore direct proteomic analysis remains one of the few options for identifying biomarkers in fluids (Veenstra et al., 2005).

Most research into serum and plasma biomarkers have focused on human samples and few are reported for ovine or bovine samples. Work using bovine samples has included the characterization of bovine serum proteome by 2-DE and MS analysis (Wait et al., 2002), a 2-DE reference map for bovine plasma (Talamo et al., 2003), the development of bovine proteome database using 2-DE and MS (D'Ambrosio et al., 2005) and a study of protein patterns in sera of a group of heifers during pregnancy by 2-DE and MS (Cairolì et al., 2006). Optimization of techniques using bovine samples have been highlighted when available.

5.2 Challenges of plasma and serum proteomics

Serum and plasma are rich sources of protein. The challenge of biomarker discovery in serum and plasma is the division between high and low-abundance proteins, estimated to span 8-10 orders of magnitude in protein concentration. Twenty-two proteins make up 99% of the protein content of serum and plasma (Anderson and Anderson, 2002), and the remaining 1% consists of low-abundance proteins, including potential biomarkers. The approximate molar abundance and masses of high-abundance plasma proteins, adapted from Hortin et al. (2008), are summarized in Table 1. The techniques currently used in biomarker discovery, including 2-DE and LC-MS/MS, rely on the ability of MS instrumentation to accurately reflect in the output the diversity of peptide and protein components in the sample being analyzed. The selection of ions for analysis by MS, however, is not completely stochastic, but rather is dependent on the width of the chromatographic peaks or the concentration of the peptides delivered to the MS (Bell et al., 2009). There is therefore a bias against low abundance peptide ions in the analysis of a complex peptide mixture (Liu et al., 2004). The optimal range of concentrations is limited to two orders of magnitude in most MS measurements (Veenstra et al., 2005). The presence of larger molecules, including many of the

high abundance serum proteins, suppresses the signal of smaller molecules in ESI-FTMS (Stern et al., 2000).

Table 1: Approximate molar abundance and masses of polypeptides in plasma from healthy human subjects¹

¹: Adapted from Hortin et al. (2008)

Rank	Polypeptide	Concentration ($\mu\text{mol/l}$)	M_r
1	Albumin	500-800	66 400
2	Immunoglobulin κ , light chain	68-150	23 000
3	Immunoglobulin $\gamma 1$, heavy chain	40-130	52 000
4	Immunoglobulin λ , light chain	36-84	23 000
5	Immunoglobulin $\gamma 2$, heavy chain	20-90	52 000
6	Apolipoprotein A-I	30-70	28 100
7	Apolipoprotein A-II	30-60	8 700
8	Transferrin	25-45	79 000
9	α_1 -Antitrypsin	18-40	50 000
10	Immunoglobulin $\alpha 1$, heavy chain	8-50	57 000
11	Haptoglobin β -chain	6-40	35 000
12	Transthyretin subunit	15-30	13 800
13	Haptoglobin, $\alpha 1$ -chain	0-40	9 200
14	Haptoglobin, $\alpha 2$ -chain	0-40	15 900
15	α_2 HS-glycoprotein, heavy chain	9-30	38 400
16	α_2 HS-glycoprotein, light chain	9-30	4 000
17	Fibrinogen, α -chain	10-27	67 600
18	Fibrinogen, β -chain	10-27	52 300
19	Fibrinogen, γ -chain	9-24	49 000
20	Hemopexin	9-20	57 000
21	α_1 -Acid glycoprotein, gene 1	9-20	40 000
22	Immunoglobulin μ , heavy chain	4-25	70 000
23	Apolipoprotein C-III	6-20	9000
24	α_2 -Macroglobulin subunit	7-17	180 000
25	Gc-Globulin	8-14	51 000
26	Immunoglobulin $\gamma 3$, heavy chain	2-16	50 000
27	Apolipoprotein C-I	6-12	6631
28	C3, α -chain	5-10	110 000
29	C3, β -chain	5-10	75 000
30	$\alpha 1$ -Acid glycoprotein, gene 2	4-10	40 000
31	Immunoglobulin $\gamma 4$, heavy chain	0.3-13	50 000
32	α_1 -Antichymotrypsin	4-9	68 000
33	Apolipoprotein D	2-10	23 000
34	β_2 -Glycoprotein I	3-6	40 000
35	C4b-binding protein, α -chain	3-6	75 000
36	Apolipoprotein A-IV	3-6	43 000
37	Apolipoprotein C-II	2-7	8900

Rank	Polypeptide	Concentration ($\mu\text{mol/l}$)	M_r
38	Serum amyloid A4	4	13 000
39	Bikunin chain, inter- α -trypsin inhibitor	3-5	30 000
40	Immunoglobulin α_2 , heavy chain	1-7	55 000
41	Antithrombin III	3-5	65 000
42	α_1 B-Glycoprotein	3-5	63 000
43	Gelsolin	3-5	80 000
44	Ceruloplasmin	2-5	135 000
45	Factor H	2-5	155 000
46	Factor B	2-5	100 000
47	Inter- α -trypsin inhibitor, heavy chain 1	2-4	65 000
48	Inter- α -trypsin inhibitor, heavy chain 2	2-4	70 000
49	Plasminogen	2-4	90 000
50	Kininogen, heavy chain	3	60 000
51	C1 inhibitor	2-4	105 000
52	Low-molecular-weight kininogen, light chain	3	4000
53	α_1 -Microglobulin	3	27 000
54	Retinol-binding protein	2-3	21 000
55	C1q, A-chain	2-3	27 000
56	C1q, B-chain	2-3	25 000
57	C1q, C-chain	2-3	23 000

5.3 Overcoming the dynamic range of serum and plasma

In order to increase the sensitivity of current proteomic techniques, prefractionation techniques have been developed to reduce the sample complexity. Techniques include the removal of albumin using immobilized dyes, protein A/G depletion of albumin and immunoglobulins, immunoaffinity depletion of one or several high abundance proteins and ultra-filtration to remove high abundance proteins over 30 kDa. Other targeted approaches have included the use of equalizer beads (Sennels et al., 2007) or techniques used to identify particular subsets of proteins in serum, including the use of lectins for the isolation of post-translationally glycosylated proteins, cysteinyl-peptidyl enrichment, and magnetic bead separation.

Although it is clear that high abundance proteins reduce the sensitivity of current techniques in the discovery of biomarkers, it is not clear that removing

high abundance proteins will increase the likelihood of identifying biomarkers. The depletion of albumin is particularly problematic because it acts as a transport protein, binding hormones, lipids and amino acids. As a result, the depletion of albumin may also result in the specific loss of some low-abundance peptides or small proteins of interest, such as cytokines (Granger et al., 2005). Some biomarkers have been found to be associated with albumin (Gundry et al., 2009).

The success of the depletion strategy varies between the techniques used. Efficiency of depletion strategies is measured not only in terms of the percentage of the targeted protein removed, but also in terms of the removal of proteins that are non-targeted proteins. The following is an overview of techniques used to remove high abundance proteins, followed by immunoaffinity-based strategies.

Albumin, the protein with the highest relative abundance in serum/plasma, is a common target for removal. An inexpensive method of removing albumin is the use of Cibacron blue immobilized onto a matrix. Cibacron blue is a chlorotrizine dye that has high affinity for albumin (Bellei et al., 2010). There are columns that use Cibacron blue alone (Affi-gel Blue) or in combination with protein A (Aurum kit). Cibacron blue binds NAD, FAD, and ATP binding sites of proteins in addition to albumin (Prester et al., 1992; Thresher and Swaisgood, 1990) and is not completely effective at removing albumin. Ahmed et al. (2003) reported 96-98% removal of albumin with a 16 hour incubation, Zolotarjova et al. (2005) identified 60 spectra specific to albumin in the column flow-through fraction. This technique has limited benefits, as suggested by the findings reported by Ahmed et al. (2003) who did not find a significant changes in the total number of spots detected by 2-DE after treatment.

Protein G is a Streptococcal cell wall protein that has affinity for albumin and IgG. Protein G has a high affinity for mouse, rat, and human albumin (Nygren et al., 1990), but does not bind with high affinity with ovine or bovine serum albumin (Johansson et al., 2002). Baussant et al. (2005) isolated the 45 amino acid region of protein G responsible for binding albumin and immobilized it onto a matrix. The column effectively removed albumin, but it also captured small quantities of very hydrophobic proteins (apolipoproteins) in addition to albumin.

Adkins et al. (2002) used proteins A and G in combination to deplete human serum, which resulted in a 3- to 5-fold increase in the number of proteins detected in a single sample by LC-MS/MS. With this increase in the number of proteins identified, some lower abundance serum proteins in the ng/ml range were identified, including human growth hormone, IL-12, and prostate-specific antigen.

More recent advancements have focused on the use of immunoaffinity depletion of high-abundance serum proteins. Initial approaches to immunoaffinity depletion were initially limited to the most abundant proteins, albumin, and IgG. Wang et al. (2003) reported an 88% depletion of serum albumin after treatment with anti-human serum albumin (α -HSA) couple to protein G, and an increase in the number of features observed on 2-DE gel. Huang et al. (2005) similarly found an increase of 48% in the number of spots detected after depletion using α -HSA and IgG. The number of spots in the low molecular weight region was increased after 5% acetonitrile was used to disrupt potential interactions between albumin and peptides. These findings, however, may not extend to the use of the columns with cattle samples, as Marco-Ramell and Bassols (2010) recently reported incomplete depletions of albumin and IgG using an immunoaffinity column using cattle serum. Some researchers are taking advantage of albumin's binding capacity to study the 'albuminome' by using monoclonal antibodies against albumin to bind full-length albumin as well as its fragments and its associated proteins (Gundry et al., 2007).

The success of the targeted removal of proteins using immunoaffinity methods has been recently expanded to include other high abundance proteins. The multiple affinity removal system (MARS) removes the six highest abundant proteins (albumin, α 1-antitrypsin, haptoglobin, transferrin, IgA, IgG). This column contains affinity-purified polyclonal antibodies (Bellei et al., 2010). The MARS column consistently depletes 98-99% of targeted proteins, as measured by ELISA (Brand et al., 2006), representing 90-95% of total serum protein (Govorukhina et al., 2006). The removal of the high abundance serum proteins allows for a 10- to 20-fold increase in the loading capacity of the remaining digested protein on the reverse phase column (Govorukhina et al., 2006), doubling

the proteins identified by 2-DE (Yocum et al., 2005) and increasing in the number of proteins detected by SELDI-TOF MS, particularly in the albumin region (Bellei et al., 2010). Martosella et al. (2005) identified 34 additional proteins in the depleted fraction, some which have serum concentrations of approximately 20 ng/ml. The column shows adequate recovery of spiked proteins in the flow-through fraction, with rates varying between 60 and 95% (Brand et al., 2006). The column has been shown to remove three proteins non-specifically, including one report of C3 fragment, apolipoprotein A-I and transthyretin binding nonspecifically (Bellei et al., 2010), another lists 194 proteins identified in the bound fraction (Gong et al., 2006), and Govorukhina et al. (2006) reported that only 19-27% of cytochrome c was recovered from the flow-through fraction. The proteins that bind to the column do so in a reproducible fashion (Brand et al., 2006).

Newer versions of the column include the MARS-7, which depletes albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin, and fibrinogen, and MARS-14, which additionally removes α 2-macroglobulin, α 1-acid glycoprotein, IgM, apolipoproteins A-I and A-II, complement C3 and transthyretin. Not all proteins were captured as efficiently with the MARS-14 column, with apolipoprotein A-I and apolipoprotein A-II captured at 47 and 31% efficiency. Treatment with these columns showed an average 4-fold enrichment (Tu et al., 2010). Twenty non-targeted proteins were identified in the bound fraction of MARS-7 and 19 were also identified using the MARS-14 column. The depletion permitted the detection of 23 proteins with serum concentrations of 10 ng/ml or lower. The authors found that the 50 most abundant plasma proteins accounted for over 90% of ions sampled, and contrasted this with the analysis of cell proteomes, where only about 30% of sampled ions map to the top 50 proteins (Tu et al., 2010). These approaches enhance the detection of high and medium abundance proteins, but do little to improve the detection of low-abundance proteins (<10 ng/ml). The concentration and sensitivity is not sufficient to reach into the range of known tumour markers.

The IgY-depletion system is similar to the MARS technology. This system also uses polyclonal antibodies, therefore these columns can tolerate modest changes in sequence homology, which allows the column to maintain good depletion efficiency across a number of species (Cellar et al., 2008). The human column (IgY-12) (Beckman Coulter) depletes 12 highly abundant serum proteins, including albumin, total IgG, α 1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α 1-glycoprotein (orosomucoid), α 2-macroglobulin, HDL (apolipoprotein A-I and A-II) and fibrinogen. The significant advantage of using chicken IgY antibodies over IgG antibodies results from the evolutionary distance between chicken and mammals, allowing for greater immunogenicity against conserved mammalian proteins such as albumin (Hinerfeld et al., 2004). When used as a preparative step for SELDI-TOF MS analysis, 6-9 new peptides were detected and the signal-to-noise ratio increased 5- to 7-fold. Roche et al. (2006) also found a modest increase in the number of peaks detected by SELDI-TOF MS, with 3 unique proteins detected in the bound fraction and 24 in the flow-through fraction. Using SELDI-TOF MS does not accurately represent the strength of this technique because of the limited binding capacity of protein array spots and large concentration differences that still exist between the remaining classical plasma proteins and low-abundance peptides and proteins (Linke et al., 2007). The results of immunodepletion combined with more sensitive technique shows promise for biomarker detection.

In a study of the human plasma proteome by Pieper et al. (2003), a multistep chromatographic procedure was set up to fractionate the plasma sample, including immunoaffinity depletion of 8 high abundance serum proteins, anion-exchange chromatography, size-exclusion chromatography, and finally separation by 2DE prior to analysis of the digested protein spots by MALDI-TOF MS or LC-MS/MS. The prefractionation allowed for detection levels of approximately 10 μ g protein/ml using CBB staining. Using this approach, they were able to identify 325 proteins from serum in the analysis of approximately 3 700 unique spots on 2DE gels. Qian et al. (2008) combined IgY-12 and Supermix, a mixture of IgY antibodies raised against the flow-through from an IgY-12 column to deplete

medium-abundance proteins and to separate approximately 60 abundant proteins from low abundance proteins in plasma. A total of 695 plasma proteins were identified using downstream LC-MS/MS analysis including 42 proteins with concentrations in the 100 pg/ml-100 ng/ml range. The use of the SuperMix alone allowed a 60-80% increase in proteome coverage when compared to IgY-12 alone. Depletion of high and medium abundance proteins therefore increases the sensitivity of detection using 2-DE MS and LC-MS/MS. Andersen et al. (2010) were able to combine IgY-12 immunodepletion, DIGE and MS/MS to identify biomarkers of ovarian cancer in depleted serum samples, which were validated by Western blotting.

Newer approaches to serum protein analysis are shifting from depletion to equalization of protein concentration in samples. ProteoMiner, for example, is based on the use of a combinatorial library of hexa-peptides coupled to beads, each being present in equal numbers (Boschetti and Righetti, 2008). Proteins are retained by the matrix through interaction with the peptide ligands; the amount of each protein that is retained is limited by the availability of the matrix-bound peptide. This reduces the high dynamic range of concentrations and allows for amplification of the low-abundance proteome (Boschetti and Righetti, 2008). This method may alter the relative abundance of proteins between samples, complicating downstream quantitation of proteins (Pernemalm et al., 2009). Macro-Rammell and Bassols (2010) reported a substantial enrichment of low abundance proteins in cattle using this approach.

Chapter 2 References

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Connecting statement 1

After the comprehensive review of the literature, the following is the first manuscript of the thesis in which proteomic profiling of the sera of sheep infected with *F. hepatica* was undertaken using SELDI-TOF MS. The study involved identification of traces associated with infection by comparison of SELDI-TOF MS and validation of the technique using immunodetection. The format of this manuscript follows the guidelines set out by the International Journal for Parasitology, where it was published.

Chapter 3:

Discovery and validation of serum biomarkers expressed over the first twelve weeks of *Fasciola hepatica* infection in sheep

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Abstract

Serum biomarkers associated with *Fasciola hepatica* infection of Corriedale sheep were analysed during the first twelve weeks of infection using surface-enhanced laser desorption ionisation time of flight mass spectrometry (SELDI-TOF MS). In the discovery phase of analysis, pooled sera collected at week 0 and at each week post- infection (p.i.) to week 12 were fractionated by anion exchange chromatography and the protein fingerprints in individual fractions were obtained in the M/z range 1.5 to 150 kDa. A total of 2302 protein peaks were identified that varied in abundance between time points following infection with peaks increasing or decreasing in intensity, or showing transient variation in intensity, during the 12 weeks of parasite challenge. In the validation phase, candidate biomarkers in sera of individual sheep at weeks 3 and 9 p.i. were analysed, identifying 100 protein peaks, many of which are small peptides <10 kDa in size: 54% of these peaks were up-regulated in intensity at week 3 or 9 p.i. Twenty six biomarkers were chosen for further study, ranging in size from 1832 to 89 823 Da: 6 biomarkers were up-regulated at weeks 3 and 9 p.i., 16 biomarkers were up-regulated only at week 9 p.i. and four biomarkers were down-regulated at week 9 p.i. Two biomarkers up-regulated at week 9 were

identified as transferrin (77.0 kDa) and Apolipoprotein A-IV (44.3 kDa), respectively. The results show that the interaction between the host and *F. hepatica* is complex with changes in biomarker patterns beginning within 3 weeks of infection and persisting to week 9-12 or showing transient changes during infection. Identification of biomarkers expressed during ovine fasciolosis may provide insights into mechanisms of pathogenesis and immunity to *Fasciola* and may assist the rational development and delivery of vaccines.

1. Introduction

Fasciolosis is a disease affecting sheep, cattle and humans caused by *Fasciola hepatica* in temperate climates and *Fasciola gigantica* in the tropics. It is estimated that 250 million sheep, 350 million cattle and 180 million humans are at risk worldwide of infection, with production losses of over 3 billion USD per year (Hillyer and Apt, 1997a; Mas-Coma et al., 1999a; Mas-Coma et al., 2005; Spithill et al., 1999). *Fasciola spp.* parasites have complex interactions with their host. The initial phase of the disease includes parasite excystment in the small intestine and migration to the liver where subsequent movement and feeding in the liver parenchyma causes extensive tissue inflammation and necrosis. This acute phase of disease occurs during the first 5 weeks of infection in sheep and, at high worm burdens, can cause death. Sexually mature parasites migrate to the bile ducts of sheep at about 8-10 weeks after infection, where they feed on the blood and duct mucosa of the host. This chronic phase is characterized by anaemia and production losses.

Proteomic studies on *Fasciola spp.* have recently expanded from the analysis of subclasses of proteins such as cathepsins (Dalton et al., 2003) and glutathione *S*-transferases (Chemale et al., 2006; Hillyer, 2005), among others, to the identification of subsets of proteins of certain parasitic products such as ES products released by adult *F. hepatica in vitro* (Jefferies et al., 2001) and a comparison between ES products detected *in vitro* and in bile of infected sheep (Morphew et al., 2007) by two-dimensional gel electrophoresis.

Recent advancements in mass spectrometry, namely the development of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), have extended our ability to unravel proteomes. Surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) allows sample binding to chemically active ProteinChip® surfaces, such as ion-exchange or immobilized metal affinity capture (IMAC) surfaces. As with MALDI, different matrices can be used to facilitate the uniform ionisation and desorption of molecules from the ProteinChip array surface (Merchant and Weinberger, 2000). SELDI technology allows the rapid and quantitative profiling of proteins in complex samples under different biological conditions and identification of markers of a biological state, termed ‘biomarkers’ (Xiao et al., 2005).

SELDI analyses were initially applied to the discovery of early diagnostic or prognostic biomarkers of cancer, such as prostate cancer (Lehrer et al., 2003; Petricoin et al., 2002a; Semmes et al., 2005a), ovarian cancer (Kozak et al., 2003; Petricoin et al., 2002b; Zhang et al., 2004b), pancreatic cancer (Koopmann et al., 2004; Rosty et al., 2002), and renal cancer (Tolson et al., 2004; Won et al., 2003; reviewed in Xiao et al., 2005). Recently, this technique has been applied to the study of serum biomarkers of infectious diseases, such as Severe Acute Respiratory Syndrome (Poon et al., 2004; Yip et al., 2005b), African Trypanosomiasis (Papadopoulos et al., 2004) and several blood borne protozoa (Ndao et al., unpublished data). Such studies have focused on identifying a distinctive configuration of circulating serum proteins that are indicative of a specific pathophysiological state, a so-called “proteomic fingerprint”.

Here, we believe we report the first proteomic study of biomarkers in serum of sheep infected with *F. hepatica*. The aim of the study was to establish the proteomic fingerprints in sheep serum at intervals during the first 12 weeks of infection with the goals of identifying diagnostic biomarkers for early parasite invasion and gaining insights into the host-parasite interactions during establishment of infection and the transition from acute to chronic infection. As acquired resistance to *Fasciola spp.* is expressed during the first few weeks of

infection we were particularly interested to define biomarkers expressed within 5 weeks of infection since these could theoretically be involved in establishment or suppression of host immunity (Piedrafita et al., 2007; 2004; Spithill et al., 1997). Our results highlight the complexity of the changes that occur in the sheep serum protein fingerprint during fasciolosis and reveal multiple biomarkers that are expressed during the acute and chronic phases of disease.

2. *Materials and methods*

2.1 Sheep serum samples

Serum samples were obtained from eight male Corriedale sheep (2 years old) housed in a paddock with an artificial water supply. The animals were purchased from a fluke-free area and shown to be free of infection by fecal analysis and ELISA using cathepsin L1 as specific antigen (Piacenza et al., 1999). The animals were from the control group of a vaccination trial carried out in Uruguay, where 8 sheep were immunized with PBS and FCA, followed 4 weeks later by Freund's incomplete adjuvant. After 2 weeks, each sheep was orally challenged with a gelatin capsule containing 300 metacercariae of *F. hepatica* and humanely slaughtered at 12 weeks p.i. Flukes in the main bile ducts and gall bladder were removed. The worm burdens (sheep number) were: 18 (#12), 19 (#24), 20 (#16), 24 (#13), 49 (#22), 50 (#25), 61 (#20) and 70 (#30). Blood was collected from all sheep prior to infection and then weekly until the time of slaughter. The serum was obtained and then stored at -80°C.

2.2 Serum fractionation

Sera were fractionated using a Ciphergen Q HyperD F strong anion exchange resin filtration plate. The filtration plate was re-equilibrated by adding 200 µl of rehydration buffer (50 mM Tris-HCl, pH 9.0) and placed on a MicroMix 5 orbital vortex (Beckman Coulter) (form 20 and amplitude 7) for 60 min at room temperature (RT). The rehydration buffer was removed by vacuum and the resin was washed four times with 200 µl rehydration buffer and 4 times with 200 µl U1 solution (1 M urea, 0.2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-

propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 9.0). Serum samples were thawed on ice and spun at high speed (17,300 g) for 5 min at RT to remove particulates. Twenty microlitres of sample were added to a v-bottom 96-well microplate (Costar Corning) with 30 μ l of U9 buffer (9 M urea, 2% CHAPS, 50 mM Tris-HCl, pH 9). The microplate was sealed and placed on a MicroMix 5 orbital vortex (form 20, amplitude 5) for 20 min at RT. Fifty microlitres of sample were added to the equilibrated resin with 50 μ l of U1 buffer. The filtration plate was sealed and placed on the MicroMix 5 orbital vortex (form 20, amplitude 7) for 30 min at RT. The fraction was collected by vacuum. One hundred microlitres of pH 9 buffer [50 mM Tris-HCl, 0.1% octyl β -D-glucopyranoside (OGP), pH 9] were added to the wells of the filtration plate using the Biomek robot automation system (Beckman Coulter), the microplate was placed on the MicroMix 5 orbital vortex at RT for 10 min and the fraction collected by vacuum. One hundred microlitres of the following buffers were added in two consecutive applications and collected by vacuum: pH 9 buffer, pH 7 buffer (50 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.1% OGP, pH 7), pH 5 buffer (100 mM sodium acetate, 0.1% OGP, pH 5), pH 4 buffer (100 mM sodium acetate, 0.1% OGP, pH 4), pH 3 buffer (50 mM sodium citrate, 0.1% OGP, pH 3), and organic wash buffer [33.3% isopropanol, 16.7% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA)]. The two 100 μ l eluants from each pH fraction were pooled, aliquoted and stored at -80°C.

2.3 Binding of fractions to ProteinChip[®] arrays

The six pH fractions of serum were profiled on a weak cation-exchange (CM10) ProteinChip[®] Array according to the manufacturer's instructions. All steps were performed at RT. Briefly, the ProteinChip[®] arrays were placed in a CIPHERgen bioprocessor (C503-0006) and washed twice with 200 μ l low-stringency binding buffer (0.1 M sodium acetate, 0.1% Triton X-100, pH 4) and placed on a multi-tube vortexer (VWR VX-2500) at speed 1 for 5 min. Each of the fractions was bound to the chip by adding 10 μ l of sample in 90 μ l of binding buffer; the bioprocessor was placed on the multi-tube vortexer for 60 min. The samples were

discarded and the ProteinChip[®] arrays washed three times with 200 µl of binding buffer, and placed on the multi-tube vortexer for 5 min and washed two times with 200 µl 1 mM HEPES pH 7.4 for 1 min. The ProteinChip[®] arrays were air-dried prior to matrix application. In the first set of analyses, samples were not spotted randomly on chips: the eight samples from one time-point were spotted on the same array. In the second analyses, the samples from different time-points were spotted randomly on ProteinChip[®] arrays.

2.4 Preparation and application of matrix

For peptide analysis (1.5-10 kDa), a 50% saturated solution of α -cyano-4-hydroxy-cinnamic acid (CHCA) was prepared by adding 200 µl of 50% ACN, 0.25% TFA to 5 mg of CHCA and placed on a tube vortex (Fisher 12-812) at high setting for 5 min, left to stand for 5 min and centrifuged for 10 min at 17,300 g. One hundred microlitres of CHCA solution were diluted to 50% saturation with 100 µl 50% ACN/0.25% TFA solution. For protein analysis (7-150 kDa), a saturated sinapinic acid (SPA) solution was similarly prepared by adding in 50% ACN/0.5% TFA solution. Matrix (0.5 µl) was added to each spot on the ProteinChip array and air-dried prior to adding an additional 0.5 µl of matrix.

2.5 SELDI-TOF MS analysis

ProteinChip[®] arrays were read using a Ciphergen PBSIIc SELDI-TOF MS reader. Profiles were collected in the ranges of 1.5-10, 7-30, and 29-150 kDa. The intensity and sensitivity of the instrument were adjusted for each of these ranges on each day of analysis. The instrument was calibrated for dataset collection using all-in-one peptide standard (Ciphergen Biosystems) when collecting data in the 1.5-10 kDa range and all-in-one protein standard (Ciphergen Biosystems) when collecting data in the 7-30 or 30-150 kDa ranges using the intensity and sensitivity of collection. Spectra from profiling experiments are an average of data from 110 laser shots.

2.6 CIPHERGEN Express software analysis

Spectra were normalised by total ion current intensity starting and ending at the M/z of the collection ranges (1.5-10, 7-30, 30-150 kDa). The spectra were aligned to a spectrum with the normalisation factor nearest 1.0. The spectra were only aligned if the percentage coefficient of variation was reduced after the alignment. Peaks from the different spectra were aligned using the cluster wizard function of CIPHERGEN Express 3.0.6 software. The peak detection was completely automated within the M/z range of analysis: peaks were automatically detected on the first pass when the signal-to-noise (S/N) ratio was 5, and the peak was 5 times the valley depth. User-detected peaks below threshold were deleted and all first-pass peaks were preserved. Clusters were created within 0.3% of M/z for each peak detected in the first pass. The clusters were completed by adding peaks with S/N of 2 and two times the valley depth. When no peaks were detected, the peak intensity was estimated at the center of the cluster. In the discovery study, peaks were screened for potential biomarkers using the following criteria: difference in intensity must be observed in two or more consecutive weeks compared with the control sample and peaks must show at least a 2-fold increase in intensity (preferably a difference of presence/absence of intensity). The peaks were inspected to determine if they were multi-charged entities. In the validation study, P -values and receiver operating characteristic (ROC) values were calculated by using the P -value wizard by comparing 2 weeks at a time. P -values below 0.05 were considered statistically significant.

2.7 Purification of 77.0kDa and 44.3kDa biomarkers

The proteins from the pH 9 anion exchange fraction in week 0 and 9 p.i. pooled samples were separated by stepwise hydrophobic fractionation at RT. RPC PolyBio beads (BioSeptra) were washed three times with 10 times bead volumes of 80% ACN/0.1% TFA for 5 min on a rotational vortex (Barnstead/ThermoLyne Lab Quake). Fifty microlitres of beads were equilibrated with 10% ACN/0.1% TFA for 5 min on the rotational mixer. The pH 9 anion-exchange fraction sample was adjusted to 10% ACN/0.1% TFA with a final volume of

500 µl, mixed using a rotational vortex for 5 min, added to the equilibrated RPC PolyBio beads and mixed for 30 min at RT. The tube was centrifuged for 1 min at 2300 g and the supernatant removed by aspiration. Bound proteins were eluted successively using this method adding 400 µl of 10%, 20%, 30%, 40%, 50%, and 60% ACN in 0.1% TFA. Proteins in each fraction were profiled by adding 5 µl of hydrophobic fraction to a normal phase (NP20) array (Ciphergen) with SPA matrix and analysed with the PBSIIc reader. The 40% ACN/0.1% TFA sample and 60% ACN/0.1% TFA sample were concentrated using SpeedVac dehydration and reconstituted in NuPAGE[®] LDS sample buffer (4x) under denaturing reducing conditions and analyzed on a 4-12% Bis-Tris NuPAGE gel using MOPS running buffer (Invitrogen) according to the manufacturer's instructions. The gels were stained using Coomassie R250 stain and the 77.2 kDa and 44.3 kDa bands excised from the 40% ACN/0.1% TFA and 60% ACN/0.1% TFA fractions, respectively, from week 9 p.i. lanes. Trypsin digestion and LC-MS/MS analysis were carried out on an Agilent micro LC connected to an ABI Q-STAR mass spectrometer, at the Sheldon Biotechnology Centre, McGill University. The resulting tryptic peptides were searched against both mammalian and other eukaryotic NCBI databases using MASCOT search engine (<http://www.matrix-science.com/>) for product ion confirmation.

2.8 Western blot analysis of biomarkers in sera

An aliquot (1µl) of pooled sheep sera (diluted 1/25 in water) or 25 ng bovine apo-transferrin (Sigma) was prepared under denaturing reducing conditions and separated using a 4-12% Bis-Tris NuPAGE gel using MOPS running buffer (Invitrogen) as per manufacturer's instructions. The gel and 0.45 µm nitrocellulose membrane were equilibrated for 15 min with transfer buffer (48 mM Tris, 39 mM glycine, 20% v/v methanol, pH 9.2). The transfer conditions were 100 V for 1 h at 4 °C using Mini Trans-blot cell (Bio-Rad). The membrane was blocked with 5% skim milk in 0.05% PBST (0.05% Tween 20 in PBS) for 1 h at RT. The membrane was incubated with 1/2000 horseradish-peroxidase conjugated sheep anti-bovine transferrin polyclonal antibody (Bethyl

Laboratories) in 0.05% skim milk overnight at 4 °C. The membrane was washed five times with 0.05% PBST for 30 s, and four times with 0.05% PBST for 5 min. Two ml of WestPico luminescent substrate (Pierce) was added to the membrane for 5 min, blotted, and the membrane exposed to photographic film (Kodak). The same method was used for individual sera samples but a 1/10 dilution of the serum was used. Immunodetection of Apo A-IV was performed essentially as described above using 2 µl pooled or individual serum. The membrane was washed with PBS three times for 5 min prior to incubation with 1/250 dilution of with rabbit anti-human Apo A-IV antibody (Atlas antibodies) in PBS overnight at 4°C. The membrane was washed three times for 5 min and incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG antibodies (Amersham) at 1/50,000 dilution for 1 h at RT for pooled samples and 1/25,000 in individual samples. The membrane was washed and developed as described above.

2.9 Densitometry analysis

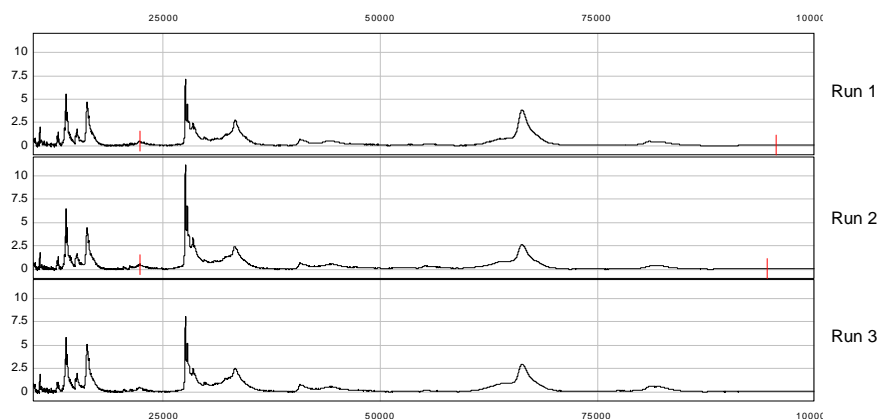
The photographic film from the Western blot analysis was scanned using a Molecular imager FX (Bio-Rad) and the bands of interest were quantitated using Quantity One 4.4.1 software (Bio-Rad) using the densitometry function grey type. The density of each of the bands from the individual sera analysis (CNT/mm²) was compared using GraphPad Prism software 4 (GraphPad Software) using a one-tailed parametric *t*-test after testing the data for normality. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1 Reproducibility study

Aliquots of pooled sera from eight sheep collected at week 1 p.i. were independently fractionated three times. The organic wash fractions were each bound to CM10 ProteinChip® arrays at the same time and analysed by SELDI-TOF MS on the same day under identical conditions. Seventeen peaks were detected in the range of 10-100 kDa in experiments 1 and 2, and 15 peaks were detected in experiment 3. Fig. 3 shows the output both as a mass spectrum (Fig. 3a) or gel view (Fig. 3b). The variation between samples in the number of peaks detected in the 10-100 kDa range with a S/N of at least 5 was 12% (2/17) and the mean variation in peak intensities was 13.54%. In a previous study by Semmes et al. (2005), the Coefficient of Variation for the S/N between institutions were 34-40% and peak intensity variation was found to vary from 15% to 36% for three individual peaks.

A



B

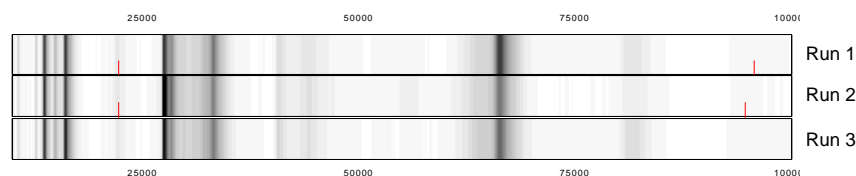


Figure 3: Reproducibility of SELDI-TOF MS analysis of sheep serum. A pool of sheep serum collected at week 1 p.i. was fractionated three times on consecutive days and then spotted and analysed on the same day by SELDI-TOF MS in the range 10-100 kDa. A) Spectral view of the three protein profiles; B) gel view of the same profiles. The tick marks indicate the peaks that were automatically detected in runs 1 and 2 but not run 3.

3.2 SELDI-TOF MS discovery study

For the discovery phase, two independent fractionations of pooled sera from each time point were performed to identify biomarkers whose patterns of expression were reproducible. Each of the anion-exchange fractions (pH 9, pH 7, pH 5, pH 4, pH 3, and organic wash) were analysed in the following calibration ranges: 1.5-10 kDa (CHCA), 7-30 kDa (SPA), and 30-150 kDa (SPA). From an analysis of all the fractions using pooled sera (data not shown) a total of 2302 clusters were identified by Ciphergen Express software (Table 2). Of these clusters, 1694 (74%) were in the range of 1.5-10 kDa, 301 (13%) in the range of 7-30 kDa and 307 (13%) in the range of 30-150 kDa. Broad patterns of variation in peak intensity are evident in the pooled sera with peaks either increasing (on) or decreasing (off) in intensity over the 12 weeks of the experiment. In addition, some peaks show transient variation in intensity (i.e. on-off-on: cluster at 145,891-48,843 Da; or off-on-off: cluster at 11,781-8656 Da, Supplementary Fig. 12a, b) during the 12 weeks of infection. Peaks were scanned for differences in intensity that occurred in two or more consecutive weeks and which demonstrated more than a 2-fold difference in intensity or presence/absence compared with the control (week 0) sample: 46 markers were targeted for further validation at weeks 3 and 9 p.i., corresponding to the early stage of migration in the liver parenchyma (week 3 p.i.) and establishment in the bile ducts (weeks 7-9 p.i.).

	1.5-10 kDa	7-30 kDa	30-150 kDa	Total
pH 9	203	80	81	364
pH 7	178	65	44	287
pH 5	563	29	38	630
pH 4	440	23	33	496
pH 3	0	14	21	35
organic wash	310	90	90	490
Total	1694	301	307	2302

Table 2: Number of clusters detected by Ciphergen Express software in all six fractions within the three M/z ranges analysed.

3.3 SELDI-TOF MS validation study

Sera from eight individual sheep collected at week 0, 3 and 9 p.i. were independently fractionated twice and analysed by SELDI- TOF MS. Figure 4a-c shows the heat maps of all 100 peaks that were found to show statistically significant differences in intensity ($P<0.05$) in both experiments in the 1.5-10 kDa, 7-30 kDa and 30-150 kDa ranges, respectively. The data were clustered to assist visualisation of patterns of variation between the different time-points. Four broad patterns of variation in peak abundance are evident: peaks that are up- or down-regulated early or late p.i., with 54% of the markers being up-regulated (Supplementary Table 4). Relative to week 0 sera, eight peaks were significantly different only at week 3 p.i., 79 peaks different only at week 9 p.i., and 13 peaks were different at weeks 3 and 9 p.i. in two independent analyses. Note that the actual number of unique markers may be less than 100 since a mass spectrum usually contains more peaks than the number of different molecular species in the sample, due to the fact that molecules form complexes or carry multiple charges and appear as several peaks in the spectrum (Dijkstra et al., 2007). These statistically significant peaks are summarised in Supplementary Table 4. Many of the 100 markers are <10 kDa in size (Supplementary Table 4).

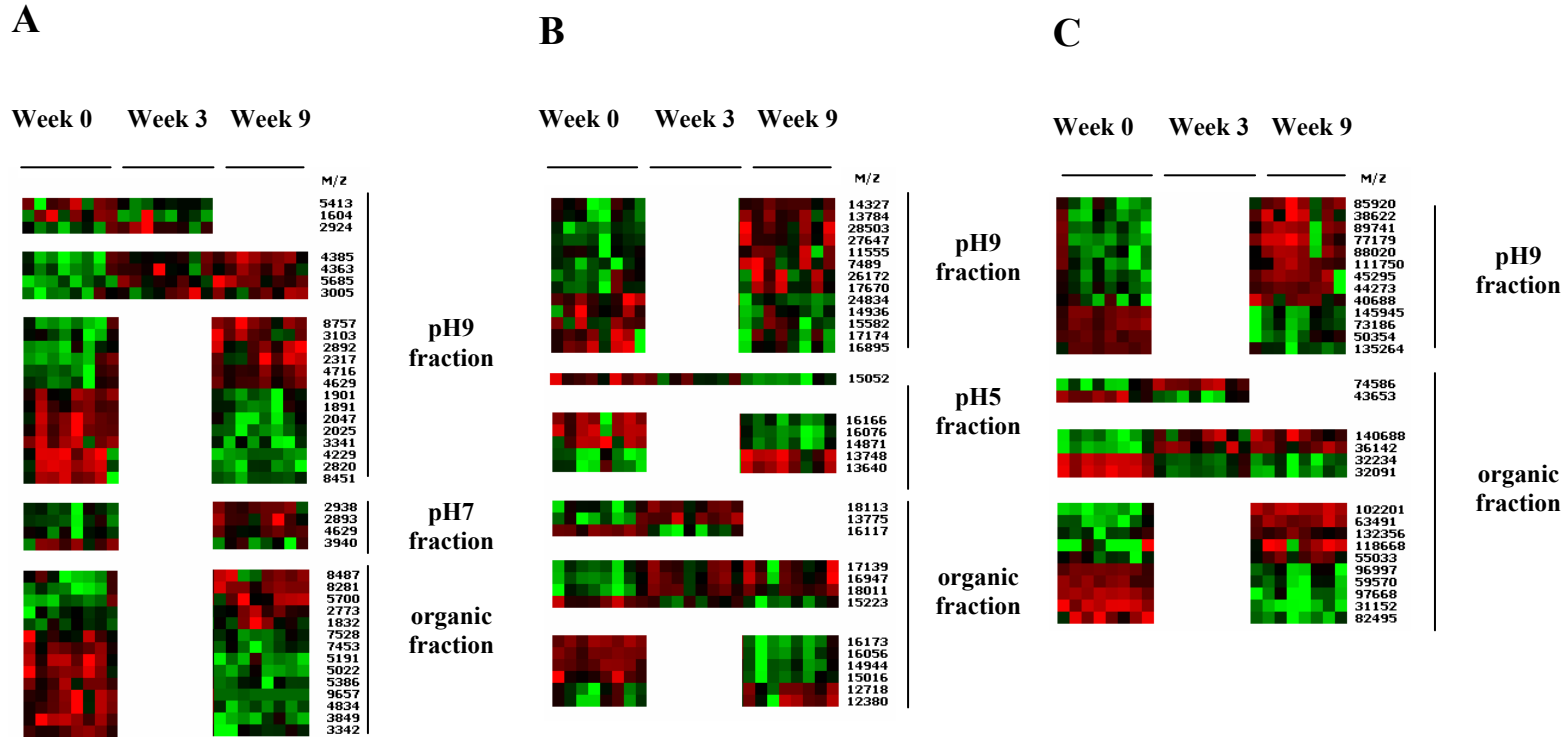


Figure 4: Heat maps of statistically significant markers of *F. hepatica* infection in sheep

Heat maps of SELDI-TOF MS profiles of single sheep sera at week 0 and weeks 3 and 9 p.i. with *Fasciola hepatica*. Heat maps of all 100 statistically significant clusters from the pH 9, pH 7, pH 5 and organic wash fractions are shown. Biomarkers found to be statistically significant in two independent analyses were determined by comparison of individual spectra from weeks 0, 3 and 9 p.i. using Ciphergen Express software. At each time-point the data are presented in rank order of increasing worm burden (sheep 12, 24, 16, 13, 22, 25, 20 and 30). Data are shown in the range 1.5-10 kDa (A), 7-30 kDa (B) and 30-150 kDa (C). Heat maps are derived from analysis of randomly spotted samples. Peaks are clustered using dendograms (not shown) to reveal patterns of upregulation (red) and down-regulation (green).

Twenty-six of the markers targeted in the discovery phase were statistically validated at weeks 3 and 9 p.i. and considered validated biomarkers: 13 biomarkers in the 1.5-10 kDa fraction (Fig. 5a), 5 biomarkers in the 7-30 kDa range (Fig. 5b), and eight biomarkers in the 30-150 kDa range (Fig. 5c). Note that biomarkers at 3341/3342 Da and 4629 Da are found in two different fractions (pH 9/organic wash and pH 9/pH 7, respectively) and may represent duplications of the same biomarker: in this case, we have validated a total of 24 biomarkers. A summary of the *P*-value statistics and corresponding ROC values of these 26 biomarkers are included in Table 2. ROC values represent the joint values of the true positive ratio (sensitivity) and the false positive ratio (1-specificity) for the relative intensity of the biomarker. ROC area values close to 1 or 0 indicate that the test has high sensitivity and specificity, whereas a value close to 0.5 indicates the test cannot reliably distinguish between positive and negative cases. Biomarkers with ROC area values nearing 1 are positively associated with infection (up-regulated), whereas values nearing 0 are negatively associated with infection (down-regulated). As shown in Table 3, six biomarkers were found to be significantly increased at week 3 and 9 p.i., 16 biomarkers were significantly increased only at week 9 p.i. and four biomarkers were significantly decreased only at week 9 p.i.

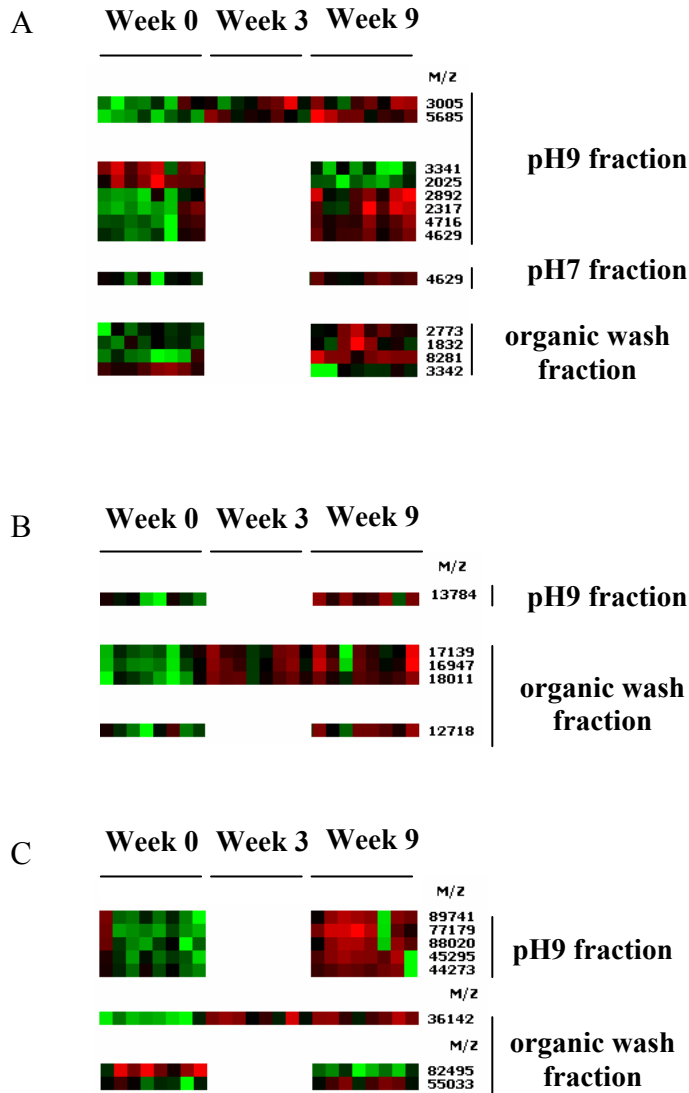


Figure 5: Heat maps of validated biomarkers of *F. hepatica* infection in sheep

Heat maps of SELDI-TOF MS profiles of single sheep sera at week 0 and weeks 3 and 9 p.i. with *Fasciola hepatica*. At each time-point the data are presented in rank order of increasing worm burden (sheep 12, 24, 16, 13, 22, 20 and 30). Heat maps show the validated biomarkers from the pH 9, pH 7 and organic wash fractions in the range 1.5-10 kDa (A), 7-30 kDa (B) and 30-150 kDa (C). Peaks are clustered using dendrograms to reveal patterns of up-regulation (red) and downregulation (green).

Table 3: Summary of 26 validated biomarkers

Summary of the 26 validated biomarkers identified in two separate fractionations of sheep sera ordered by M/z from both the first analysis (non-randomized spotting) and second analysis (randomized spotting) of individual serum fractions.

Fraction	M/z	1 st run		2 nd run	
		P -value	ROC	P -value	ROC
<i>Week 3</i>					
pH9	3005.7*	0.039	0.797	0.019	0.750
pH9	5684.9*	0.013	0.984	0.013	0.938
organic	16,946.5*	0.013	0.984	0.019	0.938
organic	17,138.4*	0.013	0.984	0.019	0.891
organic	18,011.0*	0.013	0.984	0.019	0.891
organic	36,144.5*	0.019	0.797	0.013	0.984
<i>Week 9</i>					
organic	1832.4	0.013	0.984	0.013	0.703
pH9	2025.2	0.019	0.063	0.013	0.016
pH9	2317.7	0.013	0.938	0.013	0.844
organic	2773.0	0.013	0.938	0.019	0.938
pH9	2892.9	0.013	0.891	0.013	0.891
pH9	3005.9*	0.013	0.891	0.013	0.891
organic	3342.9	0.013	0.063	0.013	0.016
pH9	3341.2	0.013	0.063	0.013	0.063
pH9	4629.4	0.013	0.938	0.013	0.938
pH7	4629.3	0.027	0.891	0.027	0.844
pH9	4716.2	0.013	0.891	0.013	0.891
pH9	5685.6	0.013	0.984	0.013	0.984
organic	8281.5	0.013	0.984	0.013	0.984
organic	12,718.6	0.013	0.938	0.013	0.891
pH9	13,784.7	0.013	0.844	0.019	0.891
organic	16,948.7*	0.013	0.984	0.019	0.844
organic	17,144.2*	0.013	0.984	0.027	0.844
organic	18,012.8*	0.013	0.984	0.013	0.984
organic	36,150.3*	0.013	0.984	0.013	0.984
pH9	44,273.9	0.039	0.844	0.019	0.891
pH9	45,295.6	0.039	0.844	0.013	0.938
organic	55,033.6	0.019	0.938	0.027	0.844
pH9	77,179.1	0.019	0.891	0.027	0.844
organic	82,495.9	0.019	0.063	0.013	0.016
pH9	88,020.3	0.027	0.891	0.039	0.844
pH9	89,741.3	0.027	0.859	0.039	0.844

P -values < 0.05 are considered statistically significant. Biomarkers with ROC values above 0.5 are termed up-regulated biomarkers and biomarkers with Receive Operation Characteristic (ROC) values below 0.5 are termed down-regulated biomarkers. Week 3 and week 9 biomarkers are shown. Peaks marked with * indicate that the peak is present at both weeks 3 and 9 as their M/z are within the error of the instrument (0.1%).

3.4 Biomarker screening and validation

The 2025 Da biomarker represents a down-regulated biomarker which demonstrates a decrease in intensity in pooled sera from approximately week 7 p.i. relative to week 0 p.i. sera (Fig. 6a). The intensity was significantly lowered (6.6-fold) at week 9 p.i., based on relative intensity data from individual sheep sera (Fig. 6b). The mean relative intensity decreased from 0.96 at week 0 p.i. to 0.14 at week 9 p.i.

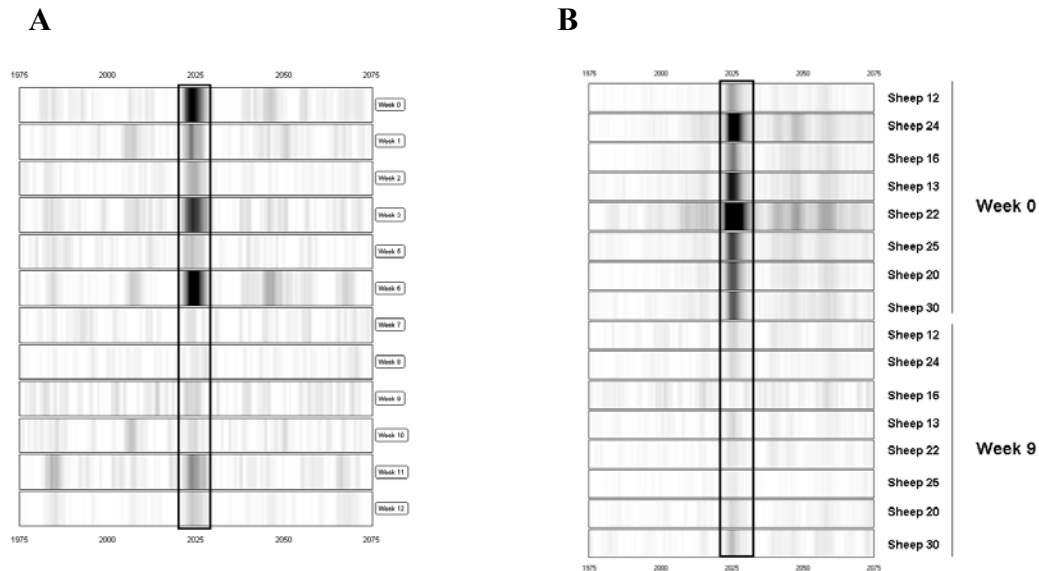


Figure 6: SELDI-TOF MS profiles of 2,025 Da biomarker

Gel view of SELDI-TOF MS profiles of the 2,025 Da biomarker in sheep sera at different times p.i. with *Fasciola hepatica*. A) 2,025 Da biomarker in pooled fractionated samples at weeks 0 through 12 p.i. (relative intensity scale of 1/-0.1). B) 2,025 Da biomarker in individual sheep at weeks 0 and 9 p.i. (relative intensity scale of 1/-0.1). At each time-point the results are presented in rank order of increasing worm burden (18-70 worms). The biomarker is boxed.

The 36.2 kDa biomarker exemplifies a biomarker with an early and persistent up-regulation of intensity in pooled sera that begins at week 1 p.i. (Fig. 7a). The relative intensity of this biomarker in individual sheep sera was significantly up-regulated 3.4-fold at week 3 p.i. and 3.6-fold at week 9 p.i. (Table 3), based on relative intensity data from individual sheep sera (Fig. 7b). The mean relative intensity increases from 0.49 at week 0 p.i. to 1.29 at week 3 p.i. and 1.51 at week 9 p.i.

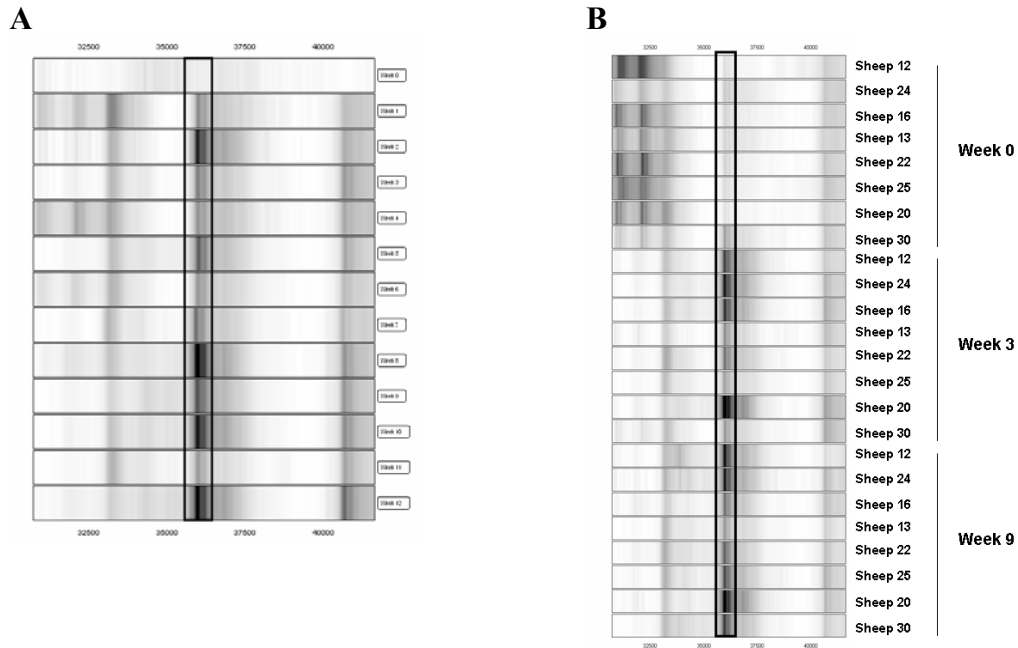


Figure 7: SELDI-TOF MS profiles of 36.2 kDa biomarker

Gel view of SELDI-TOF MS profiles of the 36.2 kDa biomarker in sheep sera at different times p.i. with *Fasciola hepatica*. A) 36.2 kDa biomarker in pooled fractionated samples at weeks 0 through 12 (relative intensity scale of 3/-0.1). B) 36.2 kDa biomarker in individual sheep at week 0 and weeks 3 and 9 p.i. (relative intensity scale of 5/-0.1). At each time-point the results are presented in rank order of increasing worm burden (18-70 worms). The biomarker is boxed.

The 44.3 kDa biomarker is an up-regulated biomarker that showed a transient increase in relative intensity in pooled sera from about weeks 4 to 9 p.i. (Fig. 8a) and was validated at week 9 p.i. (Table 3). From an analysis of individual sheep sera, the relative intensity of this biomarker was significantly elevated (3.8-fold) at week 9 p.i. (Fig 8b). The mean relative intensity increases from 0.13 at week 0 p.i. to 0.49 at week 9 p.i.

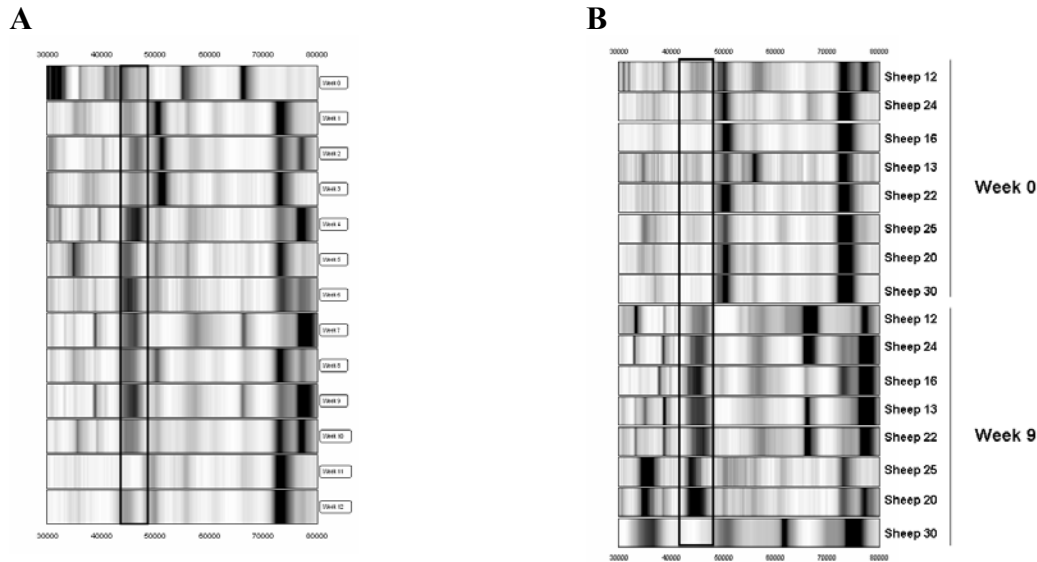


Figure 8: SELDI-TOF MS profiles of 44.3 kDa biomarker

Gel view of SELDI-TOF MS profiles of the 44.3 kDa biomarker in sheep sera at different times p.i. with *Fasciola hepatica*. A) 44.3 kDa biomarker in pooled fractionated samples at weeks 0 through 12 p.i. (relative intensity scale of 1/-0.1). B) 44.3 kDa biomarker in individual sheep at weeks 0 and 9 p.i. (relative intensity scale of 1/-0.1). At each time-point the results are presented in rank order of increasing worm burden (18-70 worms). The biomarker is boxed.

The 77.2 kDa biomarker is an up-regulated biomarker that was observed to increase in relative intensity in pooled sera during infection (Fig 9a). From an analysis of individual sheep sera, the relative intensity of the biomarker was significantly increased (5.3-fold) at week 9 p.i. as compared to week 0 sera although there was variation among the individual sheep (Fig. 9b). The mean relative intensity increased from 0.23 at week 0 p.i. to 1.21 at week 9 p.i.

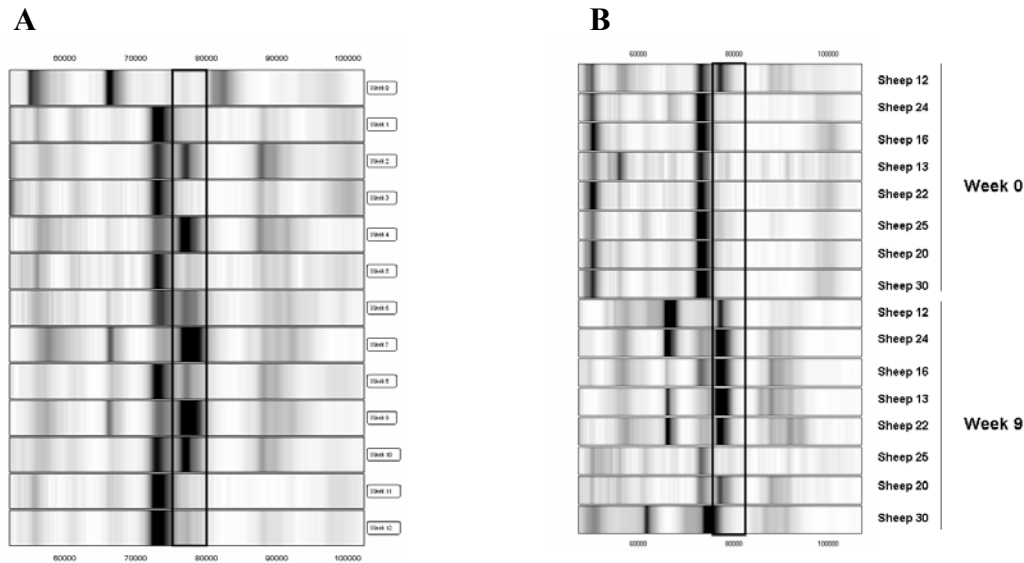


Figure 9: SELDI-TOF MS profiles of 77.2 kDa biomarker

Gel view of SELDI-TOF MS profiles of the 77.2 kDa biomarker in sheep sera at different times p.i. with *Fasciola hepatica*. A) 77.2 kDa biomarker in pooled fractionated samples at weeks 0 through 12 p.i. (relative intensity scale of 1/-0.1). B) 77.2 kDa biomarker in individual sheep at weeks 0 and 9 p.i. At each time-point the results are presented in rank order of increasing worm burden (18-70 worms). The biomarker is boxed.

3.5 Identification of biomarkers

The 77.2 kDa biomarker was further purified from the pH 9 fraction and its tryptic peptides analyzed by LC-MS/MS. One hundred and ten peptides matched to bovine transferrin (GenBank accession no. Q29443, M_r 79870 Da) with percentage coverage of 32% and a MOWSE (molecular weight search; http://www.matrix-science.com/help/scoring_help.html#MOW) score of 1164. Sixteen peptides matched to the incomplete sheep transferrin sequence with a MOWSE score of 109. The 44.3 kDa biomarker was similarly purified and its tryptic peptides analysed by LC-MS/MS. Twenty-nine peptides matched to a protein annotated as a hypothetical cattle protein (GenBank accession no. NP_001032557, M_r 42991 Da). By BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>), this protein shows 77% identity and 86% similarity to human Apolipoprotein A-IV (data not shown). The percentage coverage of the hypothetical bovine protein was 46% with a MOWSE score of 1172.

3.6 Validation of transferrin and Apo A-IV biomarkers by Western blot

In Western blot analysis with antisera to bovine transferrin, native bovine apo-transferrin appears as a doublet at about 78 and 72 kDa (Fig 10a) (Tsuji et al., 1984). The band observed in the pooled sheep samples corresponds to approximately 77 kDa and demonstrates an increase in intensity at week 9 p.i. (Fig 10a). Analysis of individual sheep serum samples at week 0 p.i. and week 9 p.i. showed that the level of transferrin varied between animals at both time points. Six sheep had an elevated level of serum transferrin by Western blotting (sheep #24, 16, 13, 22, 20 and 30) whereas no apparent change was observed in sera of sheep 12 and 25 (Fig 10b). The same six sheep also showed an apparent increase in serum transferrin peak intensity at week 9 by SELDI-TOF MS analysis (Fig. 5c, 9b). By densitometry, the intensity of the Western blot bands at 77 kDa at week 9 p.i. samples from the paired individual sera were found to be significantly increased ($P<0.05$) compared with 0 p.i., using one-tail paired parametric t-test (data not shown).

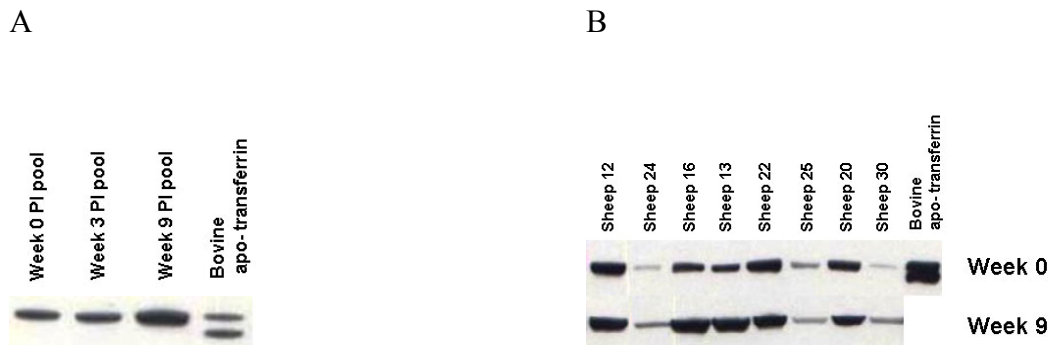


Figure 10: Western blot analysis of transferrin levels in sheep.

A) Western blot using anti-bovine transferrin of pooled sera from weeks 0, 3 and 9 p.i. with cattle apo-transferrin as a positive control. B) Western blot of 1/10 dilution of individual sera from weeks 0 and 9 p.i. Data is presented in rank order of increasing worm numbers. The worm burdens (sheep number) were: 18 (#12), 19 (#24), 20 (#16), 24 (#13), 49 (#22), 50 (#25), 61 (#20) and 70 (#30).

Western blot analysis using antisera to human Apo A-IV detected a band at approximately 44 kDa: the intensity of this 44 kDa band increased at weeks 2, 4, 6, 9, and 12 p.i. (Fig 11a). Western blotting of sera from individual sheep showed that the intensity of the 44 kDa Apo A-IV band was elevated in sheep #13, 16, 20, 22 (Fig. 11b). By densitometry, the intensity of the bands at 44 kDa of week 9 p.i. samples from the paired individual sera were found to be significantly increased ($P=0.019$) compared with week 0 p.i., using a one-tail parametric t -test (data not shown).

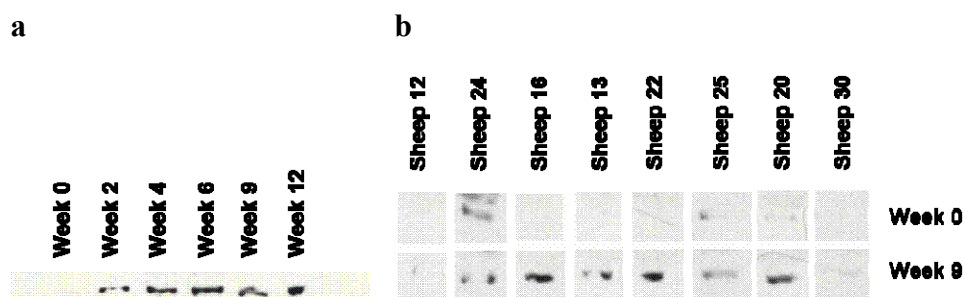


Figure 11: Western blot analysis of Apo A-IV levels in sheep.

A) Western blot using anti-human Apo A-IV of 2 μ l of pooled sera from weeks 0, 2, 4, 6, 9 and 12 p.i. with 1/50,000 secondary antibody. B) Western blot of 2 μ l of individual sera from weeks 0 and 9 p.i. with 1/25,000 secondary antibody. Data is presented in rank order of increasing worm numbers.

4. Discussion

In this study, we report for the first time an analysis of biomarkers in serial collections of sheep serum during the first 12 weeks of *F. hepatica* infection. The analysis of pooled sera provided a broad snapshot of the variation in peak intensity during infection, with peaks increasing, decreasing in intensity, or showing transient variation in intensity, during the 12 weeks of parasite challenge. It is clear that dramatic changes occur in the sheep serum proteome during fasciolosis with at least 2302 clusters shown to vary in peak intensity during infection. The two particularly informative time-points identified for further study (weeks 3 and 9 p.i.) corresponded to the migration of the parasite in the liver parenchyma and establishment in the bile ducts, respectively. From the 2302 clusters identified, 100 peaks (4.3 %) in the size range 1604-145,945 Da showed significant differences in intensity in individual sera at weeks 3 and/or 9 p.i.

relative to week 0 p.i., with a similar number of peaks being up-regulated or down-regulated. Seventy-nine peaks showed differences in intensity only at week 9 p.i. (Fig. 4; Supplementary Table 4). This bias of peaks toward later time points may reflect the impact on the serum proteome of the larger adult parasite biomass at week 9 relative to the much smaller biomass of immature parasites at week 3 p.i.

The main bottle-neck of SELDI-TOF MS profiling technology is the purification and identification of individual proteins. In order to concentrate our resources on biomarkers that are most likely to be biologically significant, a two-step approach was used. This study had the benefit of having serial bleeds from eight sheep during the course of infection so that the inherent sheep-to-sheep variation in serum protein abundance was theoretically minimised. The initial discovery phase of the study allowed us to target peaks that would be of biological relevance because they appeared in pooled sera over several consecutive weeks of infection. The second step was to validate these differences. Using the conditions that had been determined in the discovery phase, the individual sheep sera at weeks 0, 3 and 9 p.i. were fractionated and analysed. This validated the biomarkers that differed significantly between time-points and verified that the differences observed in the discovery phase were not attributable to large variations in sera from a few sheep in the group. A second independent fractionation and randomized binding experiment confirmed these results and also controlled for day-to-day differences in manipulations using the SELDI-TOF MS platform. The biomarkers were only considered to be validated if the cluster was calculated as statistically significant in both data sets.

From a total of 26 biomarkers, the validation results showed that 22 biomarkers increased in intensity and four biomarkers decreased in intensity over the first 9 weeks of infection. These biomarkers cover a wide size range (1832-89,741 Da) with many biomarkers being less than 10 kDa, suggesting that small peptides are a rich source of relevant biomarkers using the SELDI-TOF MS approach (Hortin, 2006). These results show that the interaction between the host and *F. hepatica* is complex with significant changes in biomarker patterns

beginning within 3 weeks of infection, as observed with the 36.2 kDa biomarker. The analysis of pooled sera suggests that biomarker patterns can change within 2 weeks of infection (see data for the pH 9 fraction, Supplementary Fig. 12). This complexity reflects the nature of the migration path of the parasite from the gut, through the intestinal wall, peritoneum, liver parenchyma and bile duct wall, with final residence within the bile ducts. We would anticipate different biomarkers to be generated in different host tissues or produced by different developmental stages of the parasite.

One biomarker at 77.2 kDa was identified as transferrin which increased in intensity during the biliary phase of infection at week 9 p.i. The increase in the 77.2 kDa transferrin marker at week 9 p.i. detected by SELDI-TOF MS analysis, from being almost apparently absent at week 0 p.i., is not reflected in the Western blot results where a more modest change was observed. This weak signal at week 0 p.i. in the SELDI-TOF MS spectra appears to be an artefact attributed to peak suppression by the neighbouring abundant 73.2 kDa peak, the double-charge peak of IgG (146 kDa) (Fig. 9). As observed in Supplementary Table 3, the 146 kDa and 73.2 kDa peaks decrease at week 9 p.i. (ROC=0.016, ROC=0.063/0.016 respectively). Suppression of the 77.2 kDa signal at week 0 p.i. in the SELDI analysis may account for the less drastic difference in marker intensity observed in the Western blot analysis. Peak suppression in SELDI-TOF MS analysis by blood components has been previously observed (Roche et al., 2006; van Breemen et al., 2006) and is a factor that must be carefully considered in interpretation of SELDI-TOF MS data.

Transferrin is a negative acute phase protein (McNair et al., 1998), hypothesised to decrease the bioavailability of iron to micro-organisms, whereas we observed an increase in transferrin abundance in our study. Fasciolosis, however, is characterized by both an acute (peritoneal and parenchymal phase) and a chronic (biliary) phase of disease. In a study of *F. hepatica* in sheep, with similar worm burdens to those present in our study (10-62 flukes/sheep), a loss of 9.3-23.6 ml of plasma per day was estimated and the onset of anaemia was associated with the entry of the flukes into the biliary system, which occurs at

approximately week 6 to 7 p.i. (Sinclair, 1972). Accelerated erythropoiesis occurs in response to the developing anaemia during fasciolosis (Sinclair, 1972) and a significant negative correlation has previously been observed between the concentration of haemoglobin and serum transferrin in calves (Martinsson and Mollerberg, 1973). Transferrin levels have also been shown to increase during iron deficiency in calves (Moser et al., 1994). Thus, an increase in serum transferrin levels in response to induced anaemia in sheep during fasciolosis is consistent with these observations. The variation in relative transferrin abundance between individual sheep at weeks 0 and 9 p.i. is consistent with data in cattle where transferrin concentrations have been shown to vary over a 3- to 6- fold range under different physiological conditions but the basis for this variation is not clear (McNair et al., 1998; Moser et al., 1994).

The biomarker at 44.3 kDa has been identified as Apolipoprotein A-IV, based on MS/MS. The increase in intensity of the Apo A-IV marker by SELDI-TOF MS analysis was confirmed by Western blot analysis of pooled sera where an increase in Apo A-IV abundance was observed at selected time-points from week 2-12 p.i. Apo A-IV is a 46 kDa glycoprotein and one of the minor apolipoproteins in triglyceride-rich apo B-containing lipoproteins (chylomicron, very low density proteins [VLDL]) and in Apo A-I containing lipoproteins (HDL) in blood: it is synthesised in the small intestine in humans and the liver and small intestine in rodents (Stan et al., 2003; Takahashi et al., 2004) . In cattle, Apo A-IV levels vary during lactation and decrease during fasting (Takahashi et al., 2004); variations in the intraluminal availability of bile salts may affect the regulation of Apo A-IV synthesis (Stan et al., 2003). Apo A-IV may play a role in the control of food consumption and gastric acid secretion, as well as protection against lipoprotein oxidation (Stan et al., 2003). The basis for the apparent upregulation of serum Apo A-IV during fasciolosis remains to be determined but may be related to pathological changes in the liver and bile ducts accompanying fluke infection. The regulation of Apo A-IV levels is complex and the basis for the apparent up-regulation of serum Apo A-IV during fasciolosis remains to be determined but

may be related to pathological changes in the liver and bile ducts accompanying infection.

While this manuscript was in preparation, a study was published identifying four of *F. hepatica* infection in sheep which varied in intensity between control and infected bile: chain A of the trypsin inhibitor complex (upregulated), regucalcin (downregulated), enolase 1 (downregulated) and transferrin (downregulated) (Morphew et al., 2007) (Supplementary Table 5). This apparent discrepancy between the serum and bile transferrin levels may be related to iron homeostasis during fasciolosis. The host may alter transferrin distribution in bile to enhance iron uptake from plasma. Of the other three host bile biomarkers, we observed serum biomarkers which may correspond to the chain A of the trypsin inhibitor complex (26,169 Da) and regucalcin (31,152, 32,091, 32,234 Da) since their pattern of expression, apparent M_r and elution profile correspond with the expression, M_r and pI of the bile proteins (Supplementary Table 5). Work is in progress to confirm these observations. No serum biomarker corresponded to Enolase 1. We did not detect a serum biomarker corresponding to the main up-regulated parasite biomarker in bile, isotypes of *F. hepatica* cathepsin L (observed M_r 23,588-24,497 Da, pI 4.87-6.50) (Morphew et al., 2007). This may be related to the relatively low parasite burden in the sheep in this study.

Increased aspartate aminotransferase (AST) (E.C. 2.6.1.1) and glutamate dehydrogenase (GLDH) (EC 1.4.1.2) activity in plasma is associated with the liver damage caused by fasciolosis and appear in serum at weeks 4-6 p.i. (Sexton et al., 1990). Sheep AST exists as two isoforms of 87.1 kDa (pI of 9.14) (Orlacchio et al., 1979) and 86-88.9 kDa (Campos-Cavieres and Munn, 1973). The up-regulated biomarkers at 88.0 kDa and 89.7 kDa in the pH 9 fraction observed in week 9 sera may represent AST (Fig. 4c and Table 3). Bovine GLDH is 55.4 kDa (pI 6.77) (Moon and Smith, 1973) and a 55.4 kDa biomarker was observed in the organic wash fraction in week 9 p.i. sera (Fig. 4c and Table 3). Gamma-glutamyl transferase (GGT) (EC 2.3.2.2) is a 92 kDa serum biomarker of bile duct damage in sheep (Ferre et al., 1994; Zelazo and Orlowski, 1976) but we

did not observe a biomarker of this size in serum. Work is in progress to confirm these identities.

We were interested in identifying a biomarker that could be diagnostic of early infection and whether the intensity of any biomarker was correlated with worm burden, such that a biomarker may act as a marker of intensity of infection. We identified 13 biomarkers significantly up-regulated and another seven biomarkers whose intensity was significantly down regulated, at week 3 p.i. (Supplementary Table 3). Several markers were up-regulated in all sheep at week 3, e.g. markers at 13,775 Da, 18,113 Da (Fig. 4b, organic fraction) and 74,586 Da (Fig. 4c, organic fraction). Down-regulated markers were observed at 32,091 Da and 32,234 Da (Fig. 4c, organic fraction). Although the intensity of these biomarkers changes early post-infection, further work is required to determine if these biomarkers are specific for fasciolosis. This will require comparative analysis of sera from other parasite infections of sheep and we are attempting such studies. Other changes were observed in all sheep at week 9 such as up-regulated markers at 63,491 Da, 102,201 Da (Fig. 4c, organic fraction) and down-regulated markers at 5022 Da (Fig. 4a, organic fraction), 31,152 Da, 32,091 Da and 97,688 Da (Fig. 4c, organic fraction). A regression analysis comparing biomarker intensity and worm burdens was performed on all 100 biomarkers but no significant correlations were observed in both data sets (data not shown). This may reflect the small sample size in our study as well as the relatively low worm burden observed. Biomarkers of intensity of fluke infection may be more easily identified in sera from sheep carrying a larger worm burden since the impact of such an infection on the serum proteome would be more dramatic.

Only one published study has applied SELDI-TOF MS to serum biomarker discovery in parasitic infections. This study described a serum biomarker pattern associated with human African trypanosome infections but none of the putative biomarkers were identified (Papadopoulos et al., 2004). Recent work has also applied this technology to profile human sera from Chagas disease patients to identify five biomarkers that are diagnostic for this disease (Ndao et al., unpublished observations). In this case several proteolytic fragments of proteins

were found to be useful in various diagnostic algorithms for *Trypanosoma cruzi* infection, suggesting that analysis of peptides derived from intact proteins will prove to be a rich source of diagnostic information. It is possible that some biomarkers we observed, particularly those smaller than 10 kDa, may represent fragments of larger proteins. These results demonstrate that protein profiling of parasite infection sera may reveal new insights into how parasites and hosts interact.

Future work will involve the further identification of the biomarkers validated herein to gain a fuller understanding of the effect of fasciolosis on the serum proteome. Identification of *Fasciola* and sheep biomarkers has proven to be difficult, mainly because of the low abundance of many markers (which complicates purification), the sparseness of completely annotated *Fasciola* and sheep genome sequences and the lack of homologous antibody reagents. A sheep genome project is in progress (<http://www.sheephapmap.org>) and cDNAs from *F. hepatica* are being generated (<http://www.sanger.ac.uk/Projects/Helminths/>). A focus on the less abundant proteins present in the sheep serum proteome is possible by depletion of the higher abundance proteins using the human ProteomeLab IgY-12 depletion system (Beckman Coulter, Roche et al. 2006) which is also effective at binding abundant sheep proteins (Rioux et al., unpublished data). This may allow us to further identify proteins involved in pathology and host immune responsiveness, such as biomarkers of acquired resistance to *Fasciola* or biomarkers associated with immunosuppression, which is a feature of fasciolosis (reviewed in Piedrafita et al., 2004). It is notable that certain sheep breeds can acquire resistance to *F. gigantica* but not *F. hepatica*. However, the precise nature of the responses determining acquired resistance to fluke infection is not known (Piedrafita et al., 2004). Indonesian sheep acquire resistance to *F. gigantica* within 2-4 weeks of infection and recent results suggest that a superoxide-mediated IgG dependent effector response by monocytes/macrophages and eosinophils from these sheep is effective at killing juvenile *F. gigantica* *in vitro* (Piedrafita et al., 2007). A biomarker analysis of Indonesian sheep sera within 4 weeks of infection may reveal peptides/proteins

whose expression correlates with acquired resistance, such as proteins involved in macrophage/eosinophil activation. The identification of such functional biomarkers will provide insights into host resistance pathways which may assist the design of rational delivery systems to improve the efficacy of vaccines for fasciolosis (Hillyer, 2005).

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Chapter 3 Appendix

Supplementary data

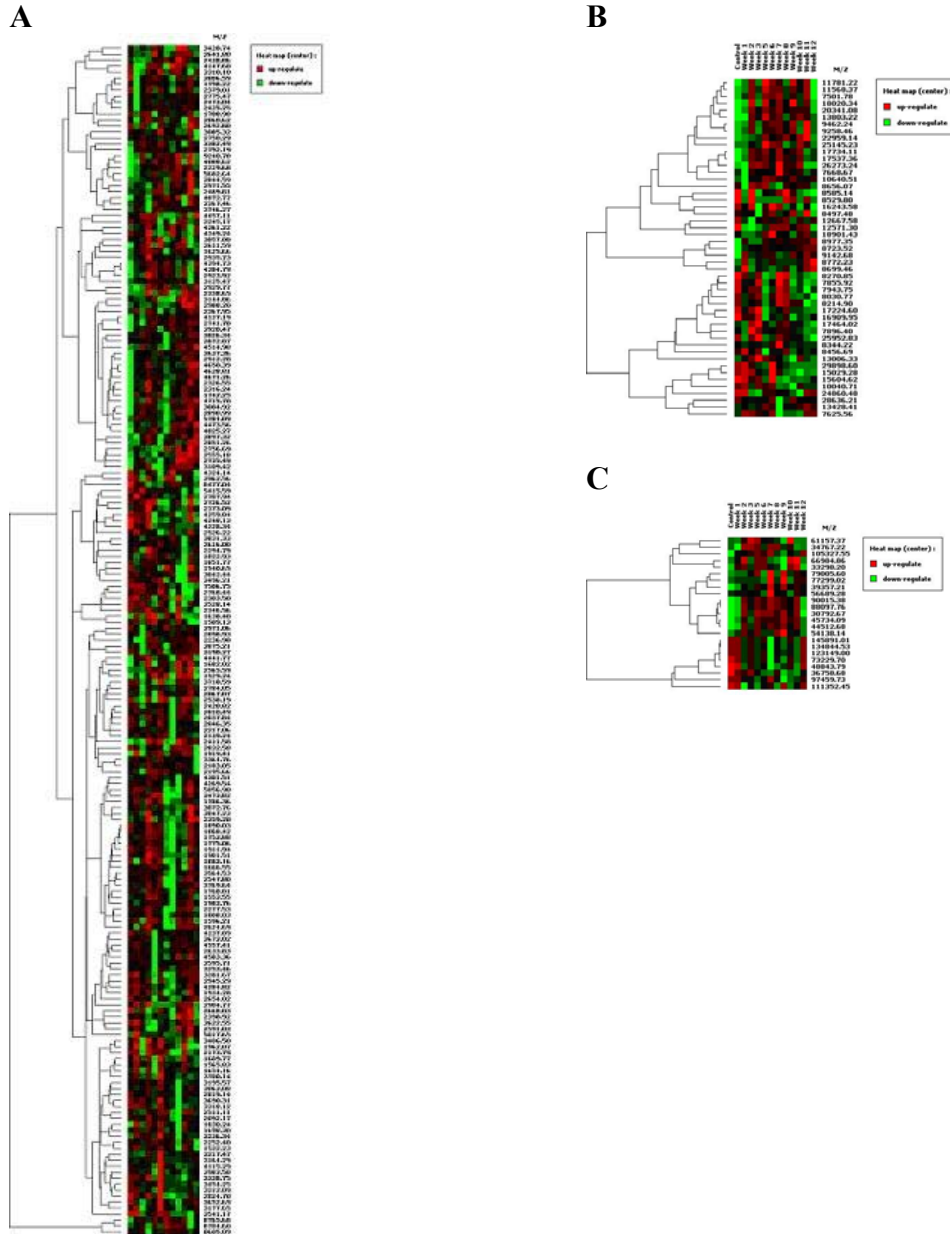


Figure 12: Heat maps of SELDI-TOF profiles of pooled sheep sera from weeks 0 to 12 p.i. with *Fasciola hepatica*. Heat maps show all clusters detected from the pH 9 fraction in the range 7-30 kDa (A) and 30-150 kDa (B). Peaks are clustered using dendrograms to reveal patterns of up-regulation (red) and down-regulation (green).

Table 4: Summary of all markers that were significantly different in two independent experiments. M/z of biomarkers in different fractions from both the first analysis of individual serum fractions (non-randomized spotting) and second analysis of individual serum fractions (randomized spotting). *P*-values <0.05 are considered statistically significant. Markers with ROC values above 0.5 are termed up-regulated biomarkers, and biomarkers with ROC values below 0.5 are termed down-regulated markers.

A

		Week 3			
		1st experiment		2nd experiment	
Fraction	m/z	p-value	ROC	p-value	ROC
pH9	1604.9	0.027	0.750	0.027	0.188
pH9	2924.7	0.039	0.891	0.039	0.797
pH9	3005.5	0.039	0.797	0.019	0.750
pH9	4363.4	0.039	0.891	0.013	0.797
pH9	4385.6	0.027	0.891	0.013	0.891
pH9	5413.9	0.027	0.250	0.039	0.250
pH9	5684.7	0.013	0.984	0.013	0.938
organic	13775.4	0.013	0.938	0.013	0.938
pH5	15056.7	0.019	0.063	0.027	0.250
organic	15223.5	0.019	0.109	0.019	0.156
organic	16117.8	0.013	0.016	0.013	0.016
organic	16946.5	0.013	0.984	0.019	0.938
organic	17138.4	0.013	0.984	0.019	0.891
organic	18011.0	0.013	0.984	0.013	0.938
organic	18113.6	0.019	0.938	0.019	0.891
organic	32091.5	0.027	0.250	0.013	0.016
organic	32234.6	0.013	0.063	0.013	0.016
organic	36141.2	0.019	0.797	0.013	0.984
organic	43653.2	0.013	0.109	0.027	0.016
organic	74586.4	0.019	0.938	0.013	0.984
organic	140597.8	0.019	0.891	0.039	0.891

B

		Week 9			
		1st experiment		2nd experiment	
Fraction	m/z	p-value	ROC	p-value	ROC
organic	1832.4	0.013	0.984	0.013	0.703
pH9	1891.3	0.027	0.063	0.013	0.063
pH9	1901.2	0.039	0.141	0.013	0.063
pH9	2025.2	0.019	0.063	0.013	0.016
pH9	2047.9	0.013	0.109	0.019	0.016
pH9	2317.7	0.013	0.938	0.013	0.844
organic	2773.0	0.013	0.938	0.019	0.938
pH9	2820.8	0.013	0.063	0.013	0.063
pH7	2893.9	0.027	0.844	0.013	0.938
pH9	2892.9	0.013	0.891	0.013	0.891
pH7	2938.1	0.013	0.984	0.013	0.938

Week 9					
Fraction	m/z	1st experiment		2nd experiment	
		p-value	ROC	p-value	ROC
pH9	3005.9	0.013	0.891	0.013	0.891
pH9	3103.2	0.019	0.891	0.013	0.844
organic	3342.9	0.013	0.063	0.013	0.016
pH9	3341.2	0.013	0.063	0.013	0.063
organic	3849.4	0.013	0.016	0.013	0.016
pH7	3940.8	0.027	0.844	0.027	0.203
pH9	4229.1	0.013	0.016	0.019	0.109
pH9	4358.6	0.027	0.938	0.039	0.844
pH9	4385.3	0.013	0.984	0.027	0.938
pH9	4629.4	0.013	0.938	0.013	0.938
pH7	4629.3	0.027	0.891	0.027	0.844
pH9	4716.2	0.013	0.891	0.013	0.891
organic	4834.3	0.013	0.016	0.013	0.016
organic	5022.8	0.019	0.063	0.013	0.016
organic	5191.3	0.039	0.109	0.013	0.063
organic	5386.7	0.013	0.109	0.013	0.063
pH9	5685.6	0.013	0.984	0.013	0.984
organic	5700.3	0.019	0.938	0.013	0.938
organic	7453.2	0.013	0.016	0.019	0.016
pH9	7489.8	0.013	0.984	0.039	0.797
organic	7528.7	0.013	0.016	0.019	0.109
organic	8281.5	0.013	0.984	0.013	0.984
pH9	8451.5	0.013	0.016	0.027	0.109
organic	8487.4	0.039	0.703	0.039	0.844
pH9	8757.9	0.027	0.938	0.013	0.984
organic	9657.4	0.013	0.016	0.013	0.016
pH9	11555.2	0.013	0.984	0.027	0.797
organic	12381.0	0.013	0.891	0.019	0.844
organic	12718.6	0.013	0.938	0.013	0.891
pH5	13640.9	0.039	0.844	0.013	0.891
pH5	13748.0	0.027	0.891	0.013	0.984
pH9	13784.7	0.013	0.844	0.019	0.891
pH9	14326.9	0.013	0.891	0.039	0.844
pH5	14871.9	0.013	0.063	0.019	0.109
organic	14944.6	0.013	0.016	0.013	0.016
pH9	14976.3	0.019	0.109	0.013	0.109
pH5	15010.0	0.013	0.016	0.013	0.016
organic	15016.2	0.013	0.063	0.013	0.016
organic	15223.5	0.013	0.063	0.013	0.016
pH9	15584.3	0.013	0.016	0.013	0.063
pH5	16076.6	0.013	0.016	0.019	0.109
organic	16056.3	0.013	0.016	0.013	0.016
pH5	16166.0	0.013	0.016	0.019	0.109
organic	16173.7	0.013	0.016	0.013	0.016
pH9	16919.4	0.013	0.016	0.027	0.203
organic	16948.7	0.013	0.984	0.019	0.844
pH9	17177.1	0.013	0.016	0.019	0.203

Week 9					
Fraction	m/z	1st experiment		2nd experiment	
		p-value	ROC	p-value	ROC
organic	17144.2	0.013	0.984	0.027	0.844
pH9	17675.9	0.019	0.891	0.013	0.891
organic	18012.8	0.013	0.984	0.013	0.984
pH9	24826.6	0.013	0.109	0.013	0.016
pH9	26169.5	0.013	0.891	0.013	0.750
pH9	27647.5	0.013	0.938	0.027	0.844
pH9	28519.2	0.013	0.938	0.013	0.844
organic	31152.4	0.013	0.109	0.013	0.016
organic	32091.5	0.019	0.109	0.013	0.016
organic	32234.6	0.013	0.016	0.013	0.016
organic	36150.3	0.013	0.984	0.013	0.984
pH9	38622.5	0.027	0.797	0.019	0.844
pH9	40688.7	0.013	0.938	0.027	0.797
pH9	44273.9	0.039	0.844	0.019	0.891
pH9	45295.6	0.039	0.844	0.019	0.891
pH9	50354.6	0.039	0.188	0.013	0.016
organic	55033.6	0.019	0.938	0.027	0.844
organic	59570.1	0.013	0.063	0.013	0.016
organic	63491.7	0.013	0.984	0.013	0.984
pH9	73186.1	0.019	0.063	0.013	0.016
pH9	77179.1	0.019	0.891	0.027	0.844
organic	82495.9	0.019	0.063	0.013	0.016
pH9	85920.2	0.013	0.938	0.027	0.891
pH9	88020.3	0.027	0.891	0.039	0.844
pH9	89741.3	0.027	0.859	0.039	0.844
organic	96997.0	0.013	0.109	0.013	0.016
organic	97668.1	0.013	0.016	0.013	0.016
organic	102201.9	0.013	0.984	0.013	0.984
pH9	111750.4	0.019	0.891	0.039	0.891
organic	118668.6	0.013	0.938	0.039	0.844
organic	132356.0	0.019	0.609	0.039	0.938
pH9	135264.1	0.027	0.063	0.019	0.016
organic	140688.9	0.013	0.797	0.013	0.984
pH9	145945.0	0.027	0.016	0.013	0.016

Table 5: Biomarkers observed in sheep bile and serum during fasciolosis¹

¹: bile biomarker data from Morphew et al. (2007)

Bile biomarker	Change observed	Observed (predicted) Mr	Observed (predicted) pI	Serum Biomarker SELDI-TOF MS	Regulation	pH fraction observed
Chain A, trypsin inhibitor complex	increased	25429-26101 (23 305)	7.65-9.29 (8.69)	26169	increased	9
Regulcacin	decreased	31470 (33308)	5.97 (5.54)	31152 32091 32234	decreased	organic
Enolase 1	decreased	48014 (47277)	6.71 (6.44)	none		
Transferrin	decreased	80914 (77753)	6.86 6.75	77179	increased	9
Fasciola cathepsin L	increased	23588-24497 (24600-37534)	4.87-6.5 (5.01-5.79)	none		

Connecting statement 2

The previous Chapter dealt with the profiling of host proteins to gain an understanding of the markers of host response to infection and potential markers of host susceptibility. In the following Chapter, proteins released by the two causative agents of fasciolosis, *F. hepatica* and *F. gigantica*, in newly excysted juveniles and adults were identified. The protein profiles of the two species were compared to identify proteins that may be responsible for the increased virulence of *F. hepatica*.

Chapter 4:

Comparative proteomic analysis of newly excysted juvenile and adult excretory-secretory products from *Fasciola hepatica* and *Fasciola gigantica*

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Abstract

This study describes proteome profiles of NEJ and adult stage *F. hepatica* and *F. gigantica* excretory-secretory products (ESP) generated by tandem mass spectrometry, in which a total of 162 unique proteins were identified. The ESP proteomes included proteases, protease inhibitors, antioxidant defence enzymes, tegumental proteins, as well as proteins involved in cellular structure and homeostasis. The proteome profiles showed greater similarities between species than life stages, with 49% of proteins shared between species and only 24% shared between life cycles. Protease expression, including cathepsin B, cathepsin L, legumain and leucyl aminopeptidase, was developmentally regulated and species-dependent. Cathepsin L, cathepsin B and legumain peptides were abundant in NEJ samples, whereas cathepsin L was the predominant protease found in adult samples. The clade-specificity of cathepsin L peptides showed clear demarcation between NEJ and adult samples, and a species-specific clade was identified in *F. gigantica* NEJ. Adult parasites had higher relative amounts of antioxidant defence enzymes and protease inhibitors than NEJ, with higher levels of antioxidant defence enzymes in *F. gigantica* than *F. hepatica*. Thioredoxin peroxidase, enolase and peptidyl isomerase were highly abundant in all samples.

This comparative overview of the proteomes of *F. hepatica* and *F. gigantica* ESP may assist in the identification of virulence markers in *F. hepatica* and the identification of proteins suitable for fasciolosis vaccines or as targets for new chemotherapeutic agents.

Keywords: Excretory-secretory products (ESP), *Fasciola hepatica*, *Fasciola gigantica*, tandem mass spectrometry, Cathepsin L, Cathepsin B

1. Introduction

Fasciola hepatica and *Fasciola gigantica* are important veterinary parasites and the causative agents of fasciolosis, a disease generating estimated losses of over 3 billion USD per year (Spithill et al., 1999). These parasites are also increasingly being recognized as parasites of clinical importance, with an estimated 2.4 million people infected with *Fasciola* species and 180 million people at risk of infection. *F. hepatica* is found predominantly in temperate climates, including Europe, the Americas and Oceania, whereas its distribution overlaps with that of *F. gigantica* in tropical climates such as Africa and Asia (Mas-Coma, 2005).

Following ingestion of the *Fasciola* metacercariae by definitive hosts such as sheep, cattle and buffalo, the parasite excysts in the small intestine of the host and migrate through the intestinal wall and the peritoneal cavity towards the liver, where they burrow through and feed on parenchymal tissue and blood. Finally, the parasite migrates to the bile ducts where it matures to the adult form and starts to release eggs into the environment. However, *F. gigantica* is slower to develop in ruminant hosts, reaching the bile duct more than 14 weeks following infection whereas *F. hepatica* parasites establish within the bile ducts 8-10 weeks after infection (Behm and Sangster, 1999; Raadsma et al., 2007). The rapid growth of *F. hepatica* parasites at 5-8 weeks post infection (Dawes and Hughes, 1964) and the speed of its progression through the migratory phase of its life cycle is postulated by Raadsma et al. (2007) to be responsible for the greater pathogenicity of *F. hepatica* infections compared to *F. gigantica* infections in permissive hosts.

In addition to differences in their rate of development, the two species of parasites exhibit differences in their ability to establish infection in susceptible hosts. In rats, the recovery of *F. gigantica* (0-5%) is much lower than that observed with *F. hepatica* (20-30%) (Gupta, 1987; Itagaki et al., 1994; Mango et al., 1972). There is little evidence of immunological resistance of sheep to infection with *F. hepatica* when judged by worm burdens after a primary and secondary challenge (Boray, 1969; Haroun and Hillyer, 1986; Meek and Morris,

1979; Sandeman and Howell, 1981; Sinclair, 1971a, b, 1973, 1975). In contrast, significant resistance to *F. gigantica* infection has been found in certain sheep breeds (A'Gadir et al., 1987), such as Indonesian Thin Tail (ITT) sheep (Roberts et al., 1997a; Roberts et al., 1997b; Roberts et al., 1997c; Wiedosari and Copeman, 1990). A degree of resistance to *F. gigantica* infections in susceptible Merino sheep has been reported (Roberts et al., 1996). There are also several reports of lower recovery rates of *F. gigantica* parasites compared to *F. hepatica* in contemporaneous challenges in sheep (Raadsma et al., 2007; Zhang et al., 2004a; Zhang et al., 2005a; Zhang et al., 2005b) and buffalo (Zhang et al., 2005a). This variation in the ability of *Fasciola spp.* to establish infections implies that there are fundamental biochemical differences between the two species in their ability to either resist or modulate host protective immune responses: unravelling the biochemical basis for these differences would be informative (Piedrafita et al., 2004; Spithill et al., 1997).

Several observations suggest that the newly excysted (NEJ) or immature juvenile *Fasciola*, not the adult parasite, is the target of the acquired host immune response in the liver. Biochemical data from multiple vaccination studies in cattle and sheep showed that killing of liver flukes occurs within about 6 weeks of infection, but only after damage occurs to the liver parenchyma (Dalton et al., 1996; Hoyle et al., 2003; Piacenza et al., 1999; Piedrafita et al., 2004). The NEJ or juvenile stage of *F. gigantica* also appears to be the target of the protective immune response in resistant ITT sheep, since these sheep show less liver damage following infection and there is a reduction in the number of parasites recovered from the liver within 2-4 weeks of a secondary infection (Roberts et al., 1997b). Piedrafita et al. (2007) have shown that *F. gigantica* NEJ are susceptible to cell-mediated superoxide radical cytotoxicity *in vitro*, whereas *F. hepatica* NEJ are not, further confirming the biological differences between the two species. The authors found a higher level of expression of superoxide dismutase (SOD), an antioxidant defence enzyme, in *F. hepatica* NEJ compared to *F. gigantica* NEJ, suggesting that such differences may be a factor in determining the resistance of *F. hepatica* NEJ to cell mediated cytotoxicity. In addition, there are differences in

the immune interactions between hosts and the two *Fasciola spp.* Zhang et al. (2005b) found that sheep peripheral blood mononuclear cells, isolated in the first three weeks of *F. gigantica* infection and then stimulated with *F. gigantica* adult excretory-secretory products (ESP), produced much lower levels of IL-10, a cytokine involved in the suppression of inflammatory reactive nitrogen oxide (NO) production implicated in the killing of *F. hepatica* NEJ (Piedrafita et al., 2001), compared to *F. hepatica* ESP. In addition, Zhang et al. (2004a) found an earlier and stronger immune response to antigens in *F. gigantica* adult ESP, relative to *F. hepatica* ESP, from both *F. hepatica*- and *F. gigantica*-infected Bellilois sheep, indicating that host responses to parasite antigens differ in the two parasites. *F. hepatica* suppresses IFN- γ production by cattle via induction of TGF- β and IL10, confirming that this species directly influences host responsiveness (Flynn and Mulcahy, 2008b).

Previous studies have profiled proteins in somatic *F. hepatica* NEJ (Tkalcovic et al., 1995), *F. hepatica* adult ESP *in vitro* (Jefferies et al., 2001; Morphew et al., 2007) and ESP proteins released into sheep bile *in vivo* (Morphew et al., 2007). Recently, Robinson et al. (2009) described a proteomic analysis of the secretome of the metacercariae, NEJ, immature and adult *F. hepatica*. To gain a better understanding of the underlying basis for differences in biology between *F. gigantica* and *F. hepatica*, we performed a comprehensive comparison of the protein profiles of ESP from these two parasites at the NEJ and adult stages. This analysis allows us to identify the abundant proteins secreted by the NEJ, which is thought to be the target of host protective immune attack, and compare these protein profiles with the adult stage of *F. hepatica* and *F. gigantica*. Differences in protein profiles observed between the two *Fasciola spp.* identify molecules that are potentially responsible for the differences in pathogenicity of *F. hepatica* and *F. gigantica* in their definitive hosts.

2. Materials and methods

2.1 NEJ Excretory-secretory material

F. hepatica metacercariae were obtained from Baldwin Aquatics, USA (Oregon strain). *F. gigantica* metacercariae were provided by Dr. Weiyu Zhang (College of Animal Science and Technology, China). Metacercariae were stored at 4°C. Prior to excystment, metacercariae were removed from cellophane sheets with a fine brush, washed with water three times by centrifugation at 2000 rpm for 5 min and incubated at 37°C overnight. Excystment was performed as described by Piedrafita et al (2007). Metacercariae were resuspended in 1% (w/v) pepsin A (Sigma) in 0.4% (v/v) HCl and incubated for 1 h at 37°C. Between this and subsequent incubations, metacercariae were washed three times with H₂O by centrifugation at 2000 rpm for 5 min. The metacercariae were incubated for 1 h at 37°C in 0.02 M sodium dithionite (Fluka), 1% NaHCO₃ (Sigma), and 0.8% NaCl. Metacercariae were washed and incubated for 2 h at 37°C on a rocking platform in 10 ml RPMI 1640 medium (Gibco) supplemented with 0.2% (w/v) taurocholic acid (Sigma), 10 µg ml⁻¹ gentamycin (Sigma), and 2 µg ml⁻¹ fungizone (Sigma). NEJ were separated from cysts and debris according to the method of Tielens et al. (1981b) by creating an excystment tower where the resuspended cells (top) were separated from the culture well (bottom) with a 100 µm filter mesh (Millipore UY1H02500). NEJ were collected from below the mesh and ESP collected by incubating NEJ in RPMI 1640 medium supplemented with fungizone (2 µg ml⁻¹) and gentamycin (10 µg ml⁻¹) at 37°C in an atmosphere containing 5% (v/v) CO₂ in 24-well plates at a density of 3 µl⁻¹. The medium was replaced after 4 h and the remaining medium was collected after overnight incubation. The viability of the parasites was assessed by observation with a stereomicroscope. Excystment was performed twice for both *F. hepatica* and *F. gigantica* and ESP were pooled and stored at -80°C. Once all excystments were complete, samples were thawed, supplemented with mini complete protease inhibitor (Roche 11836153001), concentrated using 5 NMWL Amicon and the protein concentration determined using Qubit protein quantitation (Invitrogen).

2.2 Adult excretory-secretory material

Adult ESP was prepared from *F. hepatica* collected from sheep livers and *F. gigantica* collected from buffalo as described (Wijffels et al., 1994a): ESP were provided by Dr. Janelle Wright (Charles Sturt University, Australia), and Dr. Weiyu Zhang (College of Animal Science and Technology, China), respectively. Samples were lyophilized prior to shipment, rehydrated in H₂O and complete mini protease inhibitor (Roche), and dialyzed against H₂O overnight using Slid-A-Lyzer dialysis cassette (Pierce). The protein concentration was determined using Qubit protein quantitation (Invitrogen).

2.3 Sample preparation, 1D electrophoresis and band excision

Samples were first TCA/acetone precipitated. Briefly, 4 volumes of ice cold 20% TCA/acetone supplemented with 20 mM DTT was added to each fraction, incubated at -20 °C for 60 minutes then centrifuged at 14 000 x g for 15 minutes. Pellets were resuspended in 80% cold acetone and washed twice with acetone. Adult *F. hepatica* and *F. gigantica* samples were precipitated a second time using TCA/water (final concentration of 16% TCA/water) followed by two acetone washes. *F. gigantica* adult ESP was subsequently treated with Clean-up kit (GE Healthcare), adjusted to 4 M urea and sonicated to dissolve particulates. The sample was divided and half (termed Fg Total) was loaded on a 1D SDS-PAGE gel after protein determination; the second half of the sample was subjected to centrifugation at 17 000 x g for 5 minutes and the supernatant (termed Fg Supernatant) was loaded on 1D SDS-PAGE gel after protein determination. Thirty µg of ESP from each sample were separated on a 2.4 cm gradient gel (7 to 15% acrylamide). The gels were stained with Coomassie Brilliant blue G (Sigma, Oakville, Ontario, Canada). Fifteen bands per lane were automatically excised (see Figure 14), with the Protein Picking Workstation ProXCISION (Perkin Elmer) set to excise 5 to 7 pieces per band, depending on the width of the lane.

2.4 Tryptic Digestion and Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) analysis

All analyses were performed at the McGill Proteomics Centre. Proteins from gel bands (5 to 7 gel pieces by band/well) were subjected to reduction, cysteine-alkylation and in-gel tryptic digestion by automation in a MassPrep Workstation (Micromass, Manchester, UK) as previously described (Moreno and Geary, 2008; Wasiak et al., 2002). Briefly, gel pieces were prewashed twice in 100 µl HPLC grade water for 10 min. Gel pieces were destained in 2-10 min incubations in 50 µl by 100 mM ammonium bicarbonate followed by addition of 50 µl 100% acetonitrile and 5 min incubation. Pieces were then incubated for an additional 5 min in 100% acetonitrile and liquid was removed. Destained and dehydrated gel pieces were reduced and alkylated by incubation in 50 µl 10 mM dithiothreitol for 30 min, followed by addition of 50 µl 55 mM iodoacetamide for 20 min and finally 100 µl 100% acetonitrile for 5 min. Gel pieces were washed in 50 µl 100 mM ammonium bicarbonate for 10 min followed by addition 50 µl 100% acetonitrile and 5 min incubation. Gel pieces were incubated twice in 50 µl 100% acetonitrile for 5 min and dried for 30 min at 37°C. Proteins were digested in-gel by adding 25 µl trypsin (6 ng/µl in 50 mM ammonium bicarbonate, Promega) and incubated for 30 min at room temperature followed by 4 h 30 min at 37°C. Peptides were initially extracted by adding 30 µl of a mix containing 1% formic acid and 2% acetonitrile and incubating at room temperature followed by two additional extractions in 12 µl of a mix of 1% formic acid and 2% acetonitrile and 12 µl 100% acetonitrile.

2.5 LC-MS/MS Analysis and Bioinformatics Data Processing

Extracted peptides were subjected to mass spectrometry (MS) analysis as previously described (Moreno and Geary, 2008). Using the Micro Well-plate sampler and Isocratic pump modules of an Agilent 1100 Series Nanoflow HPLC, 20 µl of the tryptic digest solution was injected on a Zorbax 300SB-C18 pre-column (5 mm X 0.35 µm) linked to an Agilent 1100 Series HPLC-system previously conditioned with water containing acetonitrile (3%) and formic acid

(0.1%). The sample was washed for 5 min at 15 μ l/min and subsequently the valve holding the pre-column to connect it between the NanoPump module and the peptides were flushed to the 75 μ m ID PicoFrit column (New Objective, Woburn, MA) filled with 10 cm of BioBasic C18 packing (5 μ m, 300 Å) to allow elution of the peptides towards the mass spectrometer at a flowrate of 200 nl/min. Solvent A was water (formic acid 0.1%) and solvent B was acetonitrile:water (95:5) (formic acid 0.1%). The acetonitrile concentration was first raised linearly from 10.5% to 42% in 40 min. It was increased linearly to 73.5% in 8 min, then to 100% in 12 min. The acetonitrile was held at 100% for 5 min then brought back to 10.5% for 2 min. The total cycle time was 72 min.

MS analysis was performed as described previously (Moreno and Geary, 2008). Briefly, eluted peptides were analyzed in a quadrupole time-of-flight (QToF) micro MS (Waters Micromass, Manchester, UK) equipped with a Nanosource modified with a nanospray adapter (New Objective, Woburn, MA). MS survey scan was set to 1 sec (0.1 sec interscan) and recorded from 350 to 1600 m/z. MS/MS scans were acquired from 50 to 1990 m/z, scan time was 1.35 s and the interscan interval was 0.15 s. The doubly and triply charged selected ions were selected from fragmentation with collision energies calculated using a linear curve from reference collision energies.

Tandem mass spectra raw data were transferred from the MS computer to a 50 terabytes server and automatically manipulated for generation of peaklists by employing Distiller version 2.1.0 (<http://www.matrixscience.com/distiller.htmls>) software with peak picking parameters set at 30 for Signal Noise Ratio (SNR) and at 0.6 for Correlation Threshold (CT). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot) and X! Tandem (www.thegpm.org; version 2007.01.01.1). X! Tandem was set up to search Trematoda (Taxonomy ID: 6178) (12 000 entries) and Mammal (Taxonomy ID: 40674) (707 975 entries) taxonomies in NCBI as well as a translated *Fasciola hepatica* EST database provided by Dr. Peter Brophy. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.50 Da and parent ion tolerance of 0.50 Da. The iodoacetamide derivative of cysteine was specified in

Mascot and X! Tandem as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Pyro-glu from Q of glutamine, deamidation of asparagine, methyl ester of the C-terminus, oxidation of methionine and acrylamide adduct of cysteine were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 unique peptide and 2 assigned spectra. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.6 Bioinformatic analysis

Sequences from the minimal list of proteins from the search against the *F. hepatica* database were used to perform BLASTP (Basic Local Alignment Search Tool; Altschul et al., 1997) searches against all sequences in the non-redundant NCBI database to identify similar proteins. Proteins showing similarity with significant *E*-values (<1) were accepted as providing putative homology for the purposes of identification and annotation (Moxon et al., 2010a). Keratin, pepsin and trypsin identifications were discarded, while proteins with more than one unique peptide matching were retained. Redundancy between the three database searches was eliminated by removing additional protein identifications based on identical peptide sequences. For the numerical analysis of the two *F. gigantea* adult ESP samples analyzed, the maximal number of the unique peptides for each protein identification was noted and the number of spectra assigned to a protein were combined to give one set of data.

2.7 Cathepsin L phylogenetic analysis

The most abundant cathepsin L sequence in the LC-MS/MS output, ABW75768, was used as a seed sequence to perform BLASTP (Altschul et al., 1997) searches of the NCBI non-redundant database (dated March 29th 2009) for other *Fasciola* cathepsin L-like proteases. The BLASTP program search identified 52 sequences with minimum expect values of $2e^{-59}$. *Fasciola* cathepsin L-like protease amino acid sequences were aligned using ClustalW with default parameters (MEGA v. 4.0.2; Tamura et al., 2007). The sequences were trimmed to include the mature coding region only. Incomplete sequences (ABF85681; ABF85682; Q24943; Q24945; Q24942; ABF85681; Q24946; Q24948) were omitted. Outgroups used for phylogenetic analysis were also aligned to *Fasciola* spp. mature cathepsin L-like sequences using ClustalW. Phylogenetic trees were constructed from protein alignments using both distance-based and character-based methods including: (1) bootstrapped (1000-trial) neighbour-joining method (MEGA v.4.0.2, Poisson-based calculation for protein); (2) bootstrapped (1000-trial) minimum evolution method (MEGA); and (3) maximum-parsimony algorithm (Tamura et al., 2007). All positions containing gaps and missing data were eliminated from the dataset. Protein cathepsin L-like sequences used as outgroups were similar to those used in Irving et al. (2003). These included *Bos taurus* (P25975), *Canis familiaris* (Q9GL24), *Cercopithecus aethiops* (Q9GKL8), *Drosophila melanogaster* (Q95029), *Homarus americanus* (P25784) *Homo sapiens* (P07711), *Mus musculus* (P06797), *Nephros norvegicus* (Q27708), *Rattus norvegicus* (P07154), and *Sus scrofa* (Q28944).

3. Results and Discussion

Previous studies have attempted to profile the complete proteome of *F. hepatica* somatic NEJ using N-terminal sequencing (Tkalcevic et al., 1995). Similar studies have been reported on the NEJ, immature (Robinson et al., 2009), and adult ESP proteome produced *in vitro* (Jefferies et al., 2001; Morphew et al., 2007; Robinson et al., 2009) or from infected bile *in vivo* (Morphew et al., 2007). The breadth of protein identifications has been limited by the lack of completely sequenced *Fasciola spp.* genomes, though a recent study by Robinson et al. (2009) mined the *F. hepatica* EST project (http://www.sanger.ac.uk/Projects/F_hepatica/) to identify the ES proteins from *F. hepatica* NEJ, immature and adult *F. hepatica* parasites. In this study, we profiled the proteomes of NEJ and adult stages of *F. hepatica* and *F. gigantica* ESP to identify potential markers of virulence and potential cross-reactive vaccine or chemotherapeutic targets.

3.1 Phylogenetic analysis of cathepsin L

To classify the sequences from our MS analysis, we carried out a phylogenetic analysis to classify cathepsin L sequences currently in the databases. A bootstrapped Neighbor Joining tree with distances based on the number of amino acid substitutions per site was found to best resolve the sequences (Fig. 13). Other methods, including Maximum Parsimony and Minimum Evolution, agreed with this phylogenetic topology. The tree classified the enzymes into six nominal clades labelled A through H (Fig. 13), using nomenclature similar to previous studies (Cancela et al., 2008; Irving et al., 2003). Our phylogenetic analysis confirms findings from previous studies (Irving et al., 2003; Law et al., 2003; Morphew et al., 2011), which found that cathepsin L expression is segregated into NEJ- and adult-specific clades (Fig. 13). As noted previously (Cancela et al., 2008; Irving et al., 2003), there was no segregation of cathepsin Ls from the two species of *Fasciola*, suggesting that at least some of the duplication events occurred prior to their divergence. Five of the seven cathepsin L clades contain at least one enzyme from both species (clades A-D, F), with the

two remaining clades containing single sequences (clades E, G). Morphew et al. (2011) recently proposed that sequence Q8T0W9, found to be distinct from clades C and D in our study, may represent a member of a novel clade, designated here as clade H.

The substrate binding specificity for the cathepsin L enzymes is determined by the S2 subsite of the active site that binds the P2 position of the substrate. This S2 subsite is composed of residues at position 67, 68, 133, 157, 160 and 205 (using papain numbering) which vary among members of the papain superfamily (Stack et al., 2008). Previous studies have shown that divergence within the S2 subsite at positions 67, 157 and 205 has the greatest influence on P2 binding (Stack et al., 2008). Polymorphisms within the S2 pocket have been associated with differences in substrate specificities, where recombinant FhCatL1 (clade D) cleaves hydrophobic residues in the P2 positions more readily than recombinant FhCatL2 (clade B), and recombinant FhCatL2 accommodates proline at the P2 position while these substrates are poorly cleaved by recombinant FhCatL1 (Robinson et al., 2008b; Smooker et al., 2000). FhCatL1 had therefore been implicated in the degradation of haemoglobin (Lowther et al., 2009), whereas FhCatL2 has been implicated in the degradation of collagen (Stack et al., 2008). The positions in the active site of NEJ clades are markedly different from the adult clade sequences (Table 6), particularly at positions 67, 157 and 205, suggesting that their specificities differ as well. Recent work (Norbury et al., 2010) has demonstrated that FhCatL1G (clade G) has relatively good activity against substrates with a proline in the P₂ position, a shorter half-life at physiological pH, relatively poor efficiency against haemoglobin and good efficiency against immunoglobulins, collagen, laminin and fibronectin. These differences would reflect the dynamic changes in the needs of the parasite from excystment and migration in the NEJ, migration through and feeding on parenchymal tissue in the immature stage and finally nutrient acquisition from host blood in the adult stage.

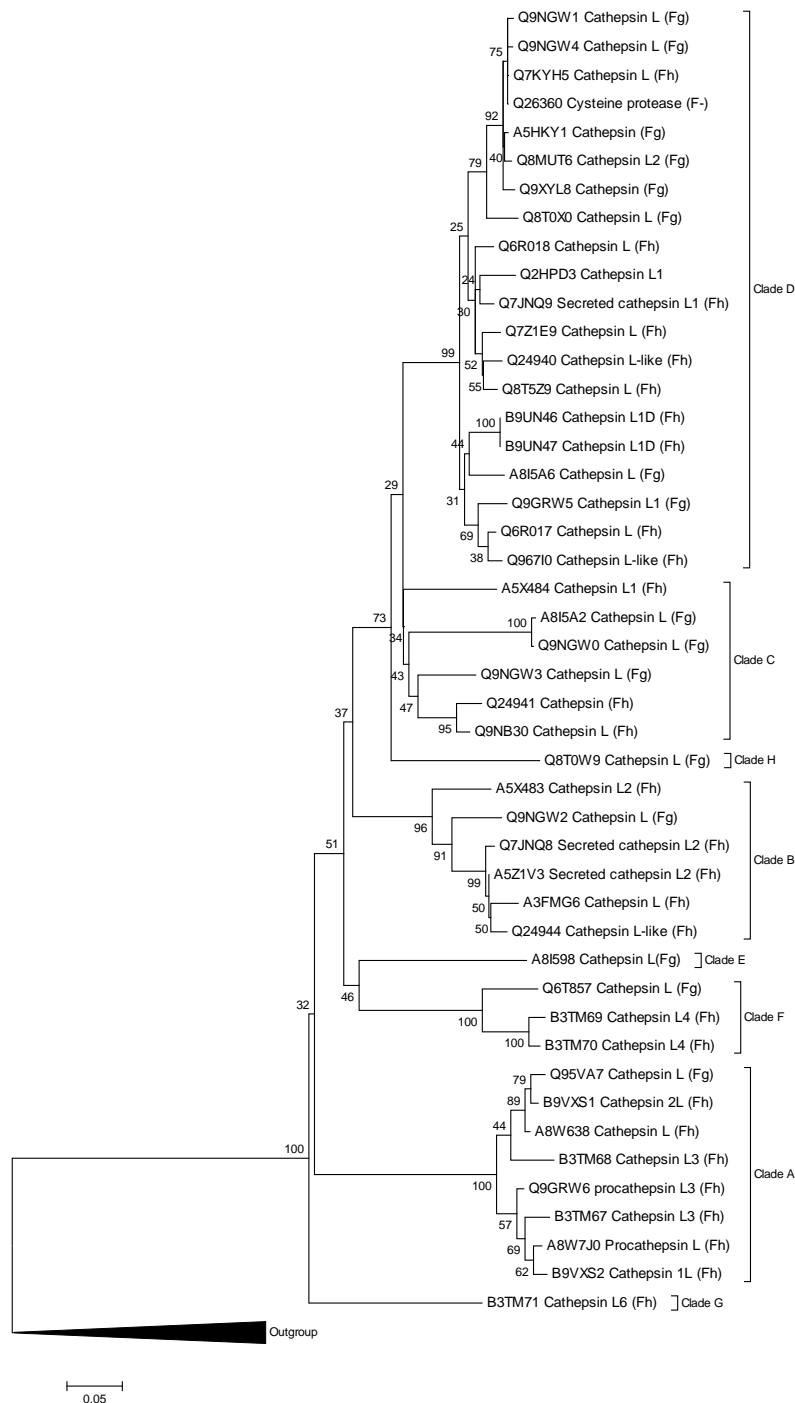


Figure 13: Evolutionary relationship of mature cathepsin L protein sequences

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987), as represented by this consensus tree of 1000 replicates (Felsenstein, 1985). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein et al. 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Proteins are identified using their UniProtKB designation.

Table 6: Cathepsin L S2 active site sequences

Residues of from the S2 active site that have been shown to contribute to differential substrate-binding in *Fasciola spp.* Cathepsin L. Residues were identified by comparison with sequences identified by Stack et al. (2008).

	67	68	133	157	158	160	205
Human cathepsin L	Leu	Met	Ala	Met	Asp	Gly	Ala
Human cathepsin K	Tyr	Met	Ala	Leu	Asn	Ala	Leu
<i>NEJ-specific clades</i>							
Clade A	Trp	Met	Ala	Val	Thr	Ala	Val
Clade F	Phe	Met	Ala	Leu	Asn	Gly/Ala	Ala/Phe
Clade G	Gly	Met	Ala	Met	Asn	Ala	Ala
<i>Adult-specific clades</i>							
Clade B	Tyr	Met	Ala	Leu	Thr	Ala	Leu
Clade C	Leu	Met	Ala	Leu	Asn	Gly	Leu/Met
Clade D	Leu	Met	Ala	Leu/Val	Asn	Ala	Leu
Clade E	Leu	Met	Ala	Leu	Asn	Ala	Leu

3.2 1DE Fractionation

The profiles seen in the SDS-PAGE fractionation of the NEJ and adult ESP showed distinct strong bands (Fig. 14), with a main band migrating at 31 kDa in the NEJ ESP samples and a main band migrating in the 22-31 kDa region in the adult ESP samples. In the NEJ ESP samples, a main band migrating near 35-40 kDa in *F. hepatica* ESP was absent in *F. gigantica* (Fig. 14A). In the adult *F. gigantica* ESP sample, a strong band below 14kDa and a minor band in the 31-45 kDa region appear to be unique to *F. gigantica*, whereas a minor band in the 45-66 kDa region appeared stronger in the adult *F. hepatica* sample (Fig. 14B).

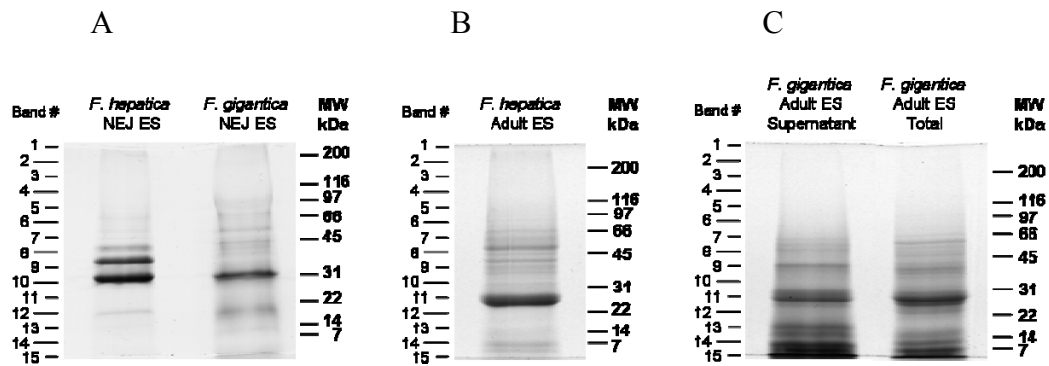


Figure 14: SDS-PAGE of NEJ and adult ESP from *F. hepatica* and *F. gigantica*

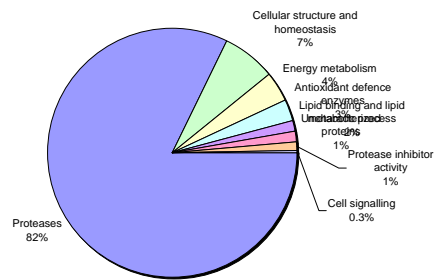
30 µg of protein from *F. hepatica* and *F. gigantica* NEJ ESP (A), *F. hepatica* adult ESP (B) and *F. gigantica* adult ESP supernatant and total extract (C), respectively, were separated by electrophoresis through a 2.4 cm gradient SDS gel (7-15%). Following staining with Coomassie Brilliant blue G, the entire lanes were subjected to automated band excision to obtain 15 pieces per lane. Proteins from gel bands were digested with trypsin and analyzed by LC-MS/MS.

3.3 Mass spectrometric analysis

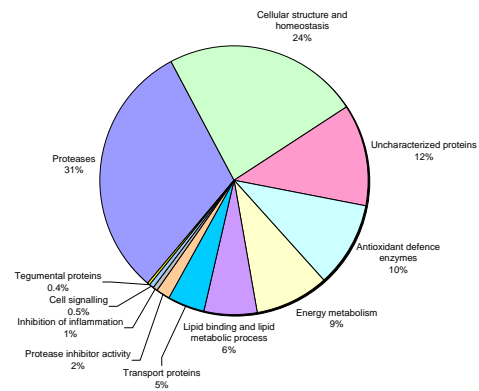
The MS analysis of the fifteen excised bands from each sample and subsequent bioinformatic analysis identified 162 unique proteins, of which 38 were found in the *F. hepatica* NEJ sample, 52 were identified in the *F. gigantica* NEJ sample, 113 in the *F. hepatica* adult sample, and 102 in the *F. gigantica* adult sample (Appendix: Table 7). The ES protein profiles of *F. hepatica* and *F. gigantica* NEJ and adults show greater similarities between the two species than between the life-stages, with 79 (49%) of the proteins shared between the two species and only 39 (24%) proteins shared between the two life cycles. The

proteins identified included proteases, protease inhibitors, antioxidant defence enzymes and tegumental proteins as well as proteins involved in energy metabolism, cellular structure and homeostasis, and lipid binding and lipid metabolic processes. Each sample had distinct proteomic profiles; the spectra identified in NEJ samples were predominantly from proteases (Fig. 15A,B), whereas the proteins in adult samples were more evenly distributed among proteases, antioxidant defence enzymes, proteins involved in energy metabolism, cellular structure and homeostasis and uncharacterized proteins (Fig. 15C,D). The relative abundance of proteases in *F. hepatica* NEJ and adult samples (82% and 31%, respectively) was roughly double the levels found in *F. gigantica*, in which 45% of NEJ spectra and 14% of adult spectra were identified as proteases (Fig. 15). Protease expression, including cathepsin B and cathepsin L clades, legumain and leucyl aminopeptidase, is developmentally regulated and species-dependent (Fig. 17 and Fig. 18). The proportion of antioxidant defence enzymes, protease inhibitors and uncharacterized proteins, on the other hand, was higher in *F. gigantica* than *F. hepatica* samples in both life stages. Despite the differences between the NEJ and adult life stages, as well as the differences between species, thioredoxin peroxidase (ABY85785), enolase (CAK47550) and peptidylprolylisomerase A (CAX72371) are all among the fifteen most abundant proteins in each of the samples analyzed (Fig. 16).

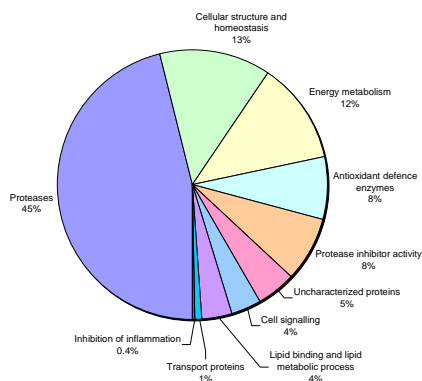
A



C



B



D

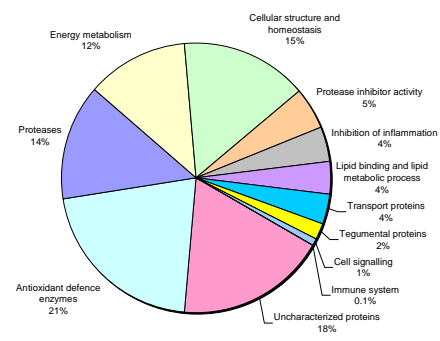


Figure 15: Relative abundance of protein identifications, by function, identified in *F. hepatica* and *F. gigantica* NEJ and adult ES

Relative abundance of proteins identified in *F. hepatica* NEJ (A), *F. gigantica* NEJ (B), *F. hepatica* adult (C) and *F. gigantica* adult (D) ESP samples according to protein classification. The percentages are based on the number of spectra assigned to individual proteins.

Fasciola hepatica NEJ

Fasciola hepatica Adult

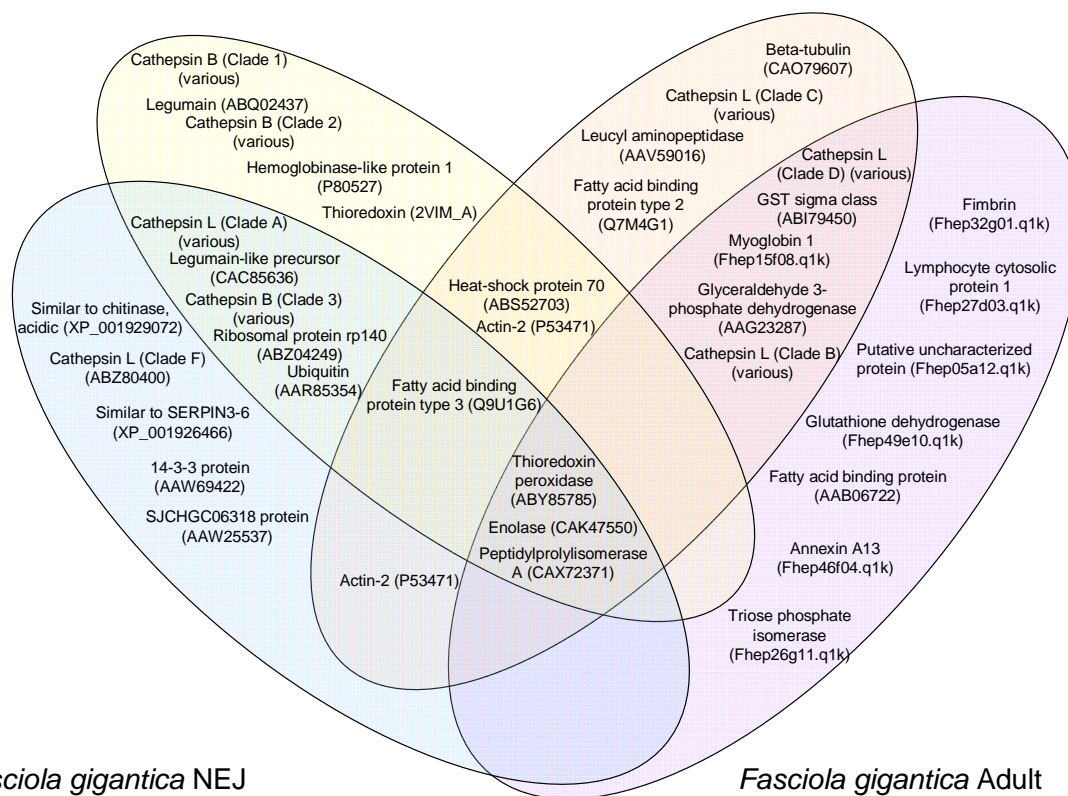


Figure 16: Fifteen most abundant proteins in *Fasciola* spp. ESP samples

A Venn diagram representing the fifteen most abundant proteins, as measured by assigned spectra, in *F. hepatica* NEJ, *F. gigantica* NEJ, *F. hepatica* adult and *F. gigantica* adult ESP samples.

3.4 Proteases

The expression of specific proteases is developmentally regulated, with distinct proteomic profiles for NEJ and adult samples (Fig. 17). Cathepsin L is the most abundant protease in all samples analyzed, but it represents a much smaller proportion of the proteases in NEJ, with 33% in *F. hepatica* NEJ ESP and 49% in *F. gigantica* NEJ ESP compared to the 72% in *F. hepatica* adult ESP and 88% in *F. gigantica* adult ESP. Cathepsin B is expressed predominantly in the NEJ stages of the parasites, with the most abundant type being classified as clade 3 cathepsin B enzymes (ABF85680, ABU62925, AAO73004) (Cancela et al., 2008). Clade 1 (ABF85678, CAD32937) and clade 2 (AAO73003) cathepsin B sequences were also identified in NEJ samples, as in other studies (Cancela et al., 2008; Law et al., 2003; Meemon et al., 2004; Wilson et al., 1998). In addition to cathepsin B in NEJ, we found a high relative expression of the asparaginyl endopeptidase legumain (CAC85636, ABQ02437), as previously reported in somatic NEJ extracts (Tkalcevic et al., 1995) and NEJ ESP (Robinson et al., 2009). Legumain isotypes showed marked developmental differences, notably a NEJ-specific sequence (CAC85636), and adult-specific sequence (ABQ02438), and a sequence common to both stages (ABQ02437). A short peptide sequence with high relative expression in *F. hepatica* NEJ ESP (Fig. 16), hemoglobinase-like protein 1 (P81527), was initially identified by N-terminal sequencing of somatic *F. hepatica* NEJ extracts (Tkalcevic et al., 1995) and corresponds by BLAST analysis to the NEJ-specific legumain (CAC85636) sequence. Beckham et al. (2006) demonstrated that a recombinant *Schistosoma mansoni* asparaginyl endopeptidase (legumain SmAE) can increase the rate of activation of FhcatB1 (AAO73003), a clade 2 enzyme, *in vitro*. The high proportionate expression of legumain CAC85636 in both *F. hepatica* and *F. gigantica* NEJ samples relative to clade 3 cathepsin B sequences suggests that this enzyme may be involved in the initial cascade of proteolytic activation at this stage of host invasion, as has been suggested (Cancela et al., 2008). Recently, McGonigle et al. (2008) showed that FhcatB1 enzyme function was necessary for penetration of the rat gut by *F. hepatica* NEJ; further study may find a similar function attributed to clade 3

cathepsin B enzymes. Clades 1 (CAD32937) and 3 (ABF85680, ABU6295) cathepsin B sequences were also detected in adult ESP samples; a cathepsin B first identified in adults (CAA81451) (Heussler and Dobbelaere, 1994) was detected in both *F. gigantica* and *F. hepatica* adult ESP only. These differences in cathepsin B expression in NEJ and adults may be attributed to the localization of cathepsin B to the gut lumen and secretory granules within the gut epithelia of juvenile flukes in NEJ, whereas it is localized to the parenchyma in adult flukes (Creaney et al., 1996). Given the higher proportion of clade 3 cathepsin B sequences in NEJ ESP samples, these should be the focus of further study for the development of an early stage vaccine, rather than the FhCatB1 enzyme that has been the subject of intense study (Smooker et al., 2010).

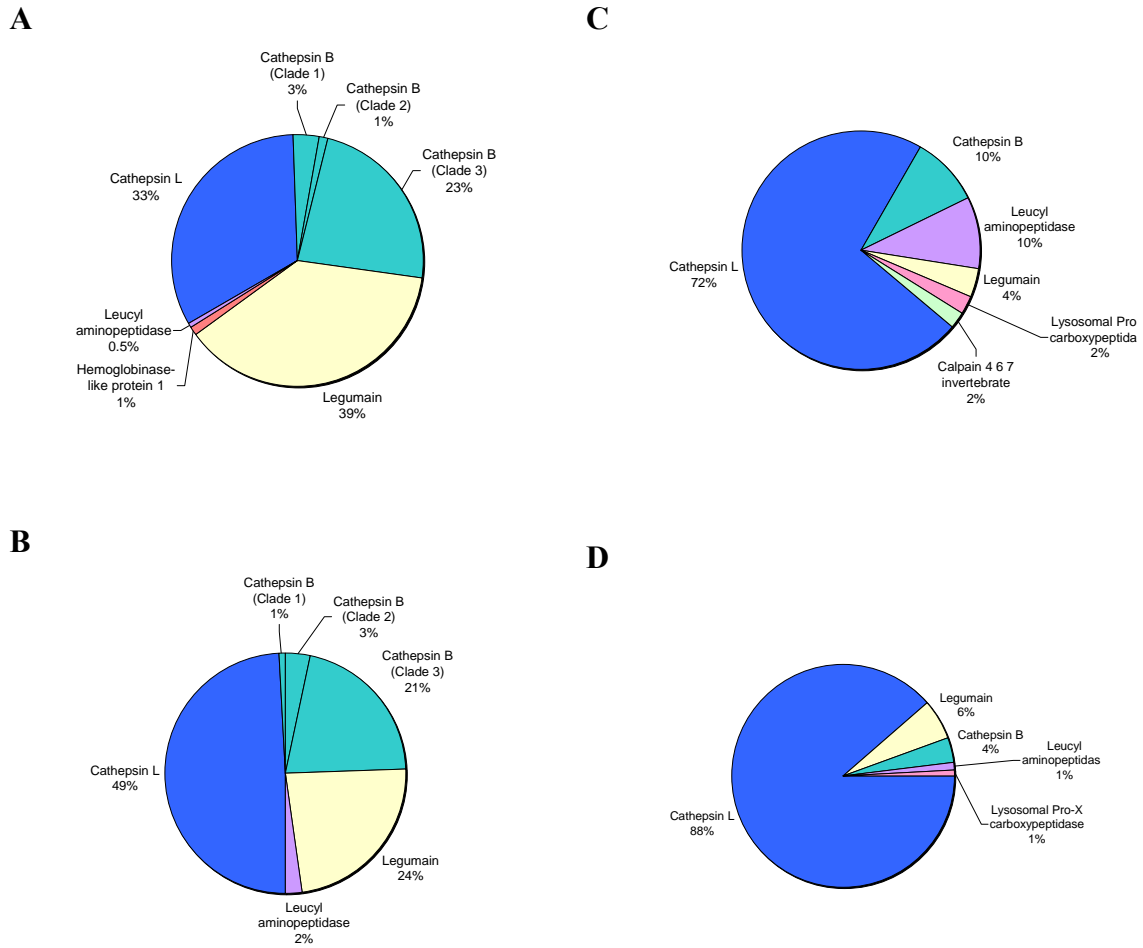


Figure 17: Protease expression in *F. hepatica* and *F. gigantica* NEJ and adult ES

Proportion of protease sequences in *F. hepatica* NEJ (A), *F. gigantica* NEJ (B), *F. hepatica* adult (C) and *F. gigantica* adult samples (D), as measured by assigned spectra.

The clade-specificity of the cathepsin L sequences was assessed by mapping LC-MS/MS results to a phylogenetic tree of mature protein-coding sequences. Cathepsin L expression shows remarkable stage- and species-specificity, as seen in Figure 18. A clear delineation of stage-specific expression is common to both *F. hepatica* and *F. gigantica*: clades A, F and G are NEJ-specific whereas clades B, C, D and E are adult-specific. Clade F cathepsin L (ABZ80400) is only expressed in *F. gigantica* NEJ, with the clade F-specific peptide EFGYVTEVK (Mascot ion score 43.2) found exclusively in the *F. gigantica* dataset. Clade F of our phylogenetic analysis (Fig. 13) includes two *F. hepatica* mRNA sequences, but the expression and/or release of these cathepsin L sequences may be limited. The difference in clade F cathepsin L sequence expression may be important since this clade has very distinct S2 position sequences: it is the only clade with Phe in the 67 position and the sequences at position 205 (Ala or Phe) also differ from the sequences in clades A through E (Table 6). Further studies may clarify if this difference translates into a difference in substrate binding affinities and its effects on the pathogenicity of *F. gigantica* NEJ. In *F. hepatica* NEJ samples, the clade A sequences represent 98% of the cathepsin L sequences and clade G sequences represent only 2%, whereas in *F. gigantica* NEJ, the clade F sequences (16%) replace a proportion of the clade A sequences, with clade G sequences being expressed in the same proportion as *F. hepatica* NEJ (2%) (Fig. 18). In the adult ESP samples, the overall distribution of sequences among clades was relatively similar, with approximately two-thirds of the spectra designated clade D, approximately one-sixth designated clades B and C, respectively, and a small minority of sequences contributed by clade E (4% in *F. hepatica*, 1% in *F. gigantica*) (Fig. 18). Previous phylogenetic analyses (Morphew et al., 2011; Robinson et al., 2008a; Robinson et al., 2008b) have suggested that the adult-specific clade D may contain a sub-clade that is *F. gigantica*-specific, termed 1C. Though *F. gigantica* adults have higher relative expression of such a sub-clade specific sequence (AAB30089) (data not shown), two peptides in the *F. hepatica* adult dataset, VTDYYTVHSGSEVELK (Mascot ion score 67.9) and NSWGSSWGER (Mascot ion score 64.1) are specific

to the proposed clade, suggesting that the expression of this clade is not exclusive to *F. gigantica*. Previous vaccination trials exclusively used adult cathepsin L preparations and recombinant molecules using adult-specific sequences (reviewed in: Dalton et al., 2003; Spithill et al., 1997). Though some rates of protection are quite high, reaching 72% in cattle (Mulcahy et al., 1998), the overall success rate is variable. The use of cathepsin L sequences specific to the NEJ stage, where the parasite is most susceptible to host immune attack may be a more appropriate candidate for a vaccine.

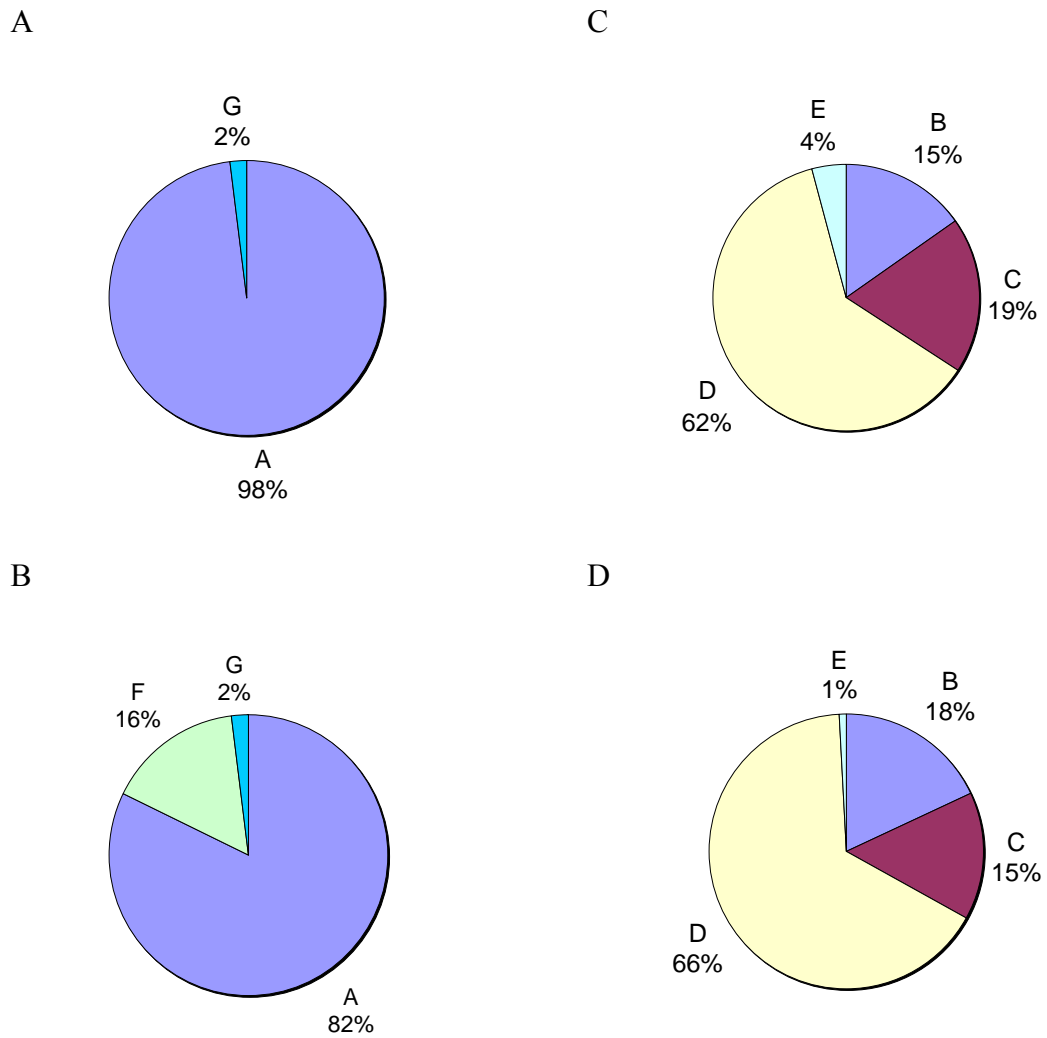


Figure 18: Cathepsin L clades expressed in *F. hepatica* and *F. gigantica* NEJ and adult ES

Clade-specificity of Cathepsin L sequences in *F. hepatica* NEJ (A), *F. gigantica* NEJ (B), *F. hepatica* adults (C) and *F. gigantica* adults (D), as measured by assigned spectra.

Leucine aminopeptidase (LAP) (AAV59016) was detected in all samples, but was highly expressed only in *F. hepatica* adults (Appendix: Table 7, Fig. 16). LAP represents 10% of the spectra assigned to protease sequences in *F. hepatica* adults, but $\leq 2\%$ of the spectra assigned to proteases in other samples (Fig. 17). LAP has been shown to be expressed in all *F. hepatica* stages by PCR (Acosta et al., 2008), but its expression has not been quantified. In *Plasmodium falciparum* infections, PfLAP participates in the haemoglobin catabolism (Skinner-Adams et al., 2010). LAP may play a similar role in *Fasciola spp.* adults, as a source of nutrients is host haemoglobin. The increased expression of *F. hepatica* LAP may confer a nutritional advantage to *F. hepatica* adults due to its increased relative abundance. LAP is immunogenic, as it is recognized by sera from fasciolosis patients (Marcilla et al., 2008). Given that LAP is expressed in all developmental stages of *F. hepatica* (Acosta et al., 2008; Carmona et al., 1994), has been found in NEJ and adult *F. gigantica* in this study, and is immunogenic in humans (Marcilla et al., 2008), this protein may be a promising vaccine candidate (Acosta et al., 2008; Piacenza et al., 1999).

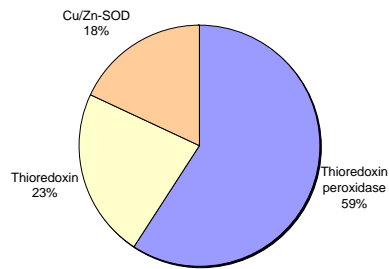
Putative lysosomal pro-x carboxypeptidase sequences (CAX71063, CAX71065 and ABJ89817) were found exclusively in adult samples (Appendix: Table 7), with peptides detected in both *F. hepatica* and *F. gigantica* adult ESP extracts, though these represent a minority of the spectra identified as proteases, with relative abundances of 2% and 1%, respectively (Fig. 17). This protease corresponds to protein SJCHGC06223, predicted to be secreted (Robinson et al., 2009), though not detected by MS/MS analysis. A putative calpain 4 6 7 invertebrate protease (XP_002582147) (Appendix: Table 7), was found exclusively in *F. hepatica* adult ESP (Fig. 17).

3.5 Antioxidant defence enzymes

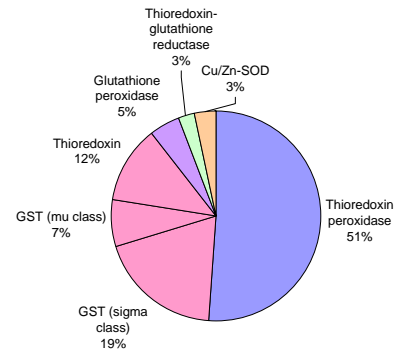
Antioxidant defence enzymes are found in all samples analyzed, with a greater abundance found in adult ESP samples. The levels of antioxidant defence enzymes in *F. gigantica* ESP samples are double the levels found in *F. hepatica* NEJ and adult stages, representing 8% and 21% of the assigned spectra in

F. gigantica, respectively (Fig. 15). Thioredoxin-related proteins were common to all samples analyzed, a group of proteins that includes thioredoxin (2VIM_A), thioredoxin-glutathione reductase (CAM96615) and thioredoxin peroxidase (ABY85785) (Fig. 19). With the exception of thioredoxin-glutathione reductase in *F. hepatica* NEJ, all proteins were detected in the samples analyzed (Fig. 19). Thioredoxin peroxidase was found in similar proportions in all samples analyzed, representing 1.6% of the assigned spectra in *F. hepatica* NEJ, 5.1% in *F. gigantica* NEJ, 5.0% in *F. hepatica* adult, and 5.8% in *F. gigantica* adult (Appendix: Table 7), ranking in the top fifteen proteins in all of the samples analyzed (Fig. 16). A previous study (Robinson et al., 2009) found thioredoxin peroxidase in *F. hepatica* NEJ and adults and thioredoxin only in *F. hepatica* adult ESP. This is the first proteomic study of ESP that has identified all three members of the thioredoxin-related enzymes involved in the detoxification of reactive oxygen species. Thioredoxin peroxidase may play a role in protecting the fluke against intracellularly produced ROS, or may function as a defence against external oxidants generated by the host immune response (McGonigle et al., 1997). Thioredoxin peroxidase has been shown to induce the recruitment of alternatively activated macrophages (Donnelly et al., 2005) and may play an important role in the bias towards a Th2 response or suppression of the immune response.

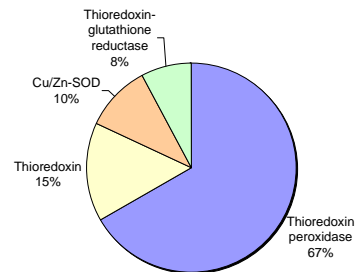
A



C



B



D

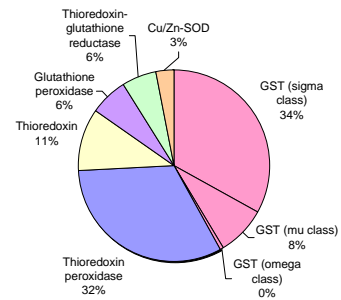


Figure 19: Antioxidant defence enzymes expressed in *F. hepatica* and *F. gigantica* NEJ and adult ESP

Proportion of antioxidant defense enzyme sequences in *F. hepatica* NEJ (A), *F. gigantica* NEJ (B), *F. hepatica* adult (C) and *F. gigantica* adult samples (D), as measured by assigned spectra.

Glutathione *S*-transferases were only found in adult samples (Appendix: Appendix: Table 7, Fig. 19). GSTs have been well characterized in *Fasciola spp.* because of their vaccine potential (Spithill et al., 1997). The GST superfamily includes phase II detoxification enzymes that catalyse the conjugation of glutathione to electrophilic substrates, such as reactive oxygen species (Sheehan et al., 2001). At least five isoenzymes have been identified in *F. hepatica*, ranging in size from 23-26.5 kDa. Four, GST 1, 7, 47 and 51, have been identified as belonging to the mu class, with GST 7, 47, and 51 being distinct from GST 1 in sequence, biochemical properties and immunogenicity (Panaccio et al., 1992). GST has been detected in *F. hepatica* adult ESP samples *in vitro* (Jefferies et al. 2001, Morphew et al. 2008), but not *in vivo* (Morphew et al., 2007). We found expression of sequences similar to isozyme 1 (2FHE_A) and isozyme 51 (ACF59730) in adult *F. hepatica* and *F. gigantica*. We also found peptides corresponding to a *F. gigantica* GST (AAD23997) that shares 94% identity with *F. hepatica* GST isozyme 51 sequence (P30112). As Chemale et al. (2006), we also found evidence of sigma and omega class GST enzymes. We found that the sigma class GST (ABI79450) is abundant in both *F. hepatica* and *F. gigantica* (Fig. 16), but the proportion is much greater in *F. gigantica* (6.6% of all assigned spectra) than *F. hepatica* (1.9% of all spectra) (Appendix: Table 7). Previous characterizations of GST enzymes have been mostly limited to the mu class of enzymes. An *Ascaridia galli* sigma class GST enzyme has been associated with prostaglandin synthesis by interacting with a GSH-dependent prostaglandin-H E-isomerase (Meyer et al., 1996; Meyer and Thomas, 1995). This enzyme may promote the endogenous synthesis of prostaglandin E in multicellular parasites. We also found a putative omega-class GST in *F. gigantica* adult ESP samples. Omega-class GSTs may act as GSH-dependent thiol transferase, removing S-thiol adducts which some proteins form with GSH and cysteine in response to oxidative stress (Sheehan et al., 2001). Omega-class GST sequences have not previously been described in *Fasciola spp.* ESP, but have recently been found in *F. hepatica* egg extracts (Moxon et al. 2010). The detection of GST only in adult samples is in line with the findings of Piedrafita et al. (2000), whereby specific

activity of GST was lower in NEJ somatic extracts than adult extracts. The increased expression of both mu- and sigma-class GST in *F. gigantica* adult ESP samples and finding omega-class GST solely in *F. gigantica* adult ESP suggests that antioxidant defence mechanisms differ between *Fasciola* spp.

Cu/Zn-superoxide dismutase (Cu/Zn-SOD) has previously been reported in *F. hepatica* adult ESP extracts *in vitro* (Jefferies et al., 2001) and *in vivo* (Morphew et al., 2007) and were detected in all samples analyzed in this study (Fig. 19). Cu/Zn-SOD represented a larger proportion of antioxidant defence enzymes in NEJ compared to adults (Fig. 19). Higher levels of Cu/Zn-SOD were observed in *F. hepatica* NEJ compared to *F. gigantica* NEJ, as a proportion of antioxidant defence enzymes, as reported previously (Piedrafita et al., 2007).

3.6 Lipid binding and lipid metabolic process

Fatty acid binding proteins are highly expressed in all samples analyzed (Fig. 16). The types of FABP expressed are developmentally-regulated, with only type 3 FABP (Q9U1G6) expressed in NEJ, and type 1 and type 2 FABP (Q7M4G0, AAB06722) specific to adults (Fig. 20). *F. hepatica* adults were unique in expressing all three types of FABP in equal proportion (Fig. 20). The relative expression of FABP is in contrast to a recent study that found all three FABP types in *F. hepatica* NEJ, immature and adult ESP extracts (Robinson et al., 2009). FABP have previously been found to be a protein complex of at least eight isoforms (Espino et al., 2001) expressed in all stages (Rodriguez-Perez et al., 1992). These isoforms differ in pI and immunological behaviour, where reactivity to Fh15 (a type 1 FABP) and a similar FABP is delayed in infected rabbits (Espino et al., 2001). As type 1 FABP were only found in adult ESP samples (Fig. 20), the delayed immunoreactivity would suggest that FABP type 1 production is also limited to adults *in vivo*. As trematodes cannot synthesize their own sterols, saturated or unsaturated fatty acids *de novo* (Meyer et al., 1970), they rely on exogenous fatty acids and lipids to make their own complex lipids. The stage-specific expression of FABPs suggests that certain FABPs play a role in the nutrient acquisition of the invading juvenile parasite.

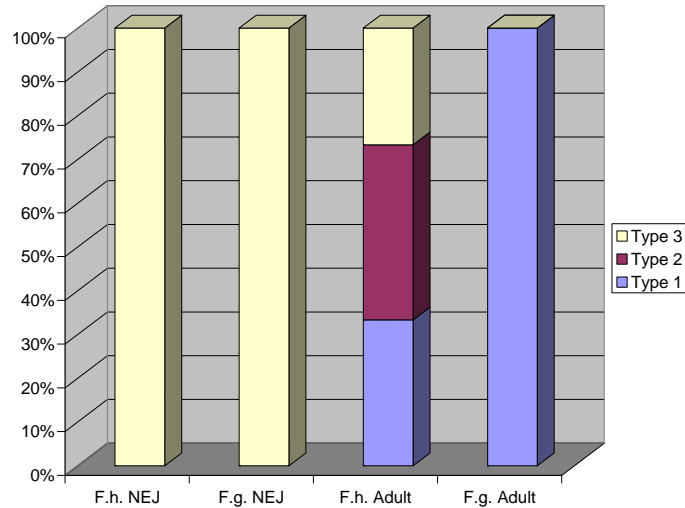


Figure 20: FABP expression in *F. hepatica* and *F. gigantica* NEJ and adult ES

Proportion of FABP sequences of types 1, 2 and 3 in *F. hepatica* NEJ, *F. gigantica* NEJ, *F. hepatica* adult and *F. gigantica* adult samples, as measured by assigned spectra.

Secreted saposin-like protein SAP-3 (ABC66278), a member of the natural-killer-lysin family, was found exclusively in *F. gigantica* adult ESP. This protein was previously identified by immunoscreening adult stage *F. gigantica* cDNA using antibodies against ESP and found to be a more abundant product in adults (Grams et al., 2006). A recent study detected SAP-3 in immature and adult *F. hepatica* ESP (Robinson et al., 2009).

3.7 Protease inhibitors

Protease inhibitors represent an important component of both NEJ and adult ESP in both *Fasciola* species, with greater amounts in both NEJ and adult *F. gigantica* ESP (Fig. 15). The protease inhibitors included both Trematoda and mammalian protease inhibitor sequences. Two Trematoda protease inhibitors were found exclusively in adult ESP samples: FH-KTM basic trypsin inhibitor (AAB46830) has previously been described as a putative serpin in *F. hepatica* (Bozas et al., 1995) and putative cys1 (CAC86126) family 2 cystatin, described by Khaznadji et al. (2005) (Appendix: Table 7). In addition to these sequences, putative stefin-1 (ACS35603) and serpin (CAX69453, CAX76360) sequences

were also identified (Appendix: Table 7). Whereas stefin-1 and one of the serpins (CAX69453) were common to all samples, a second serpin sequence (CAX76360) was found exclusively in the adult *F. gigantica* sample. Stefin-1 corresponds to an mRNA sequence recently identified in a recent study by Tarasuk et al. (2009), in which it was isolated from *F. gigantica* metacercariae and adults. This study found cystatin to be an important component of adult ESP, released in equimolar amounts with cathepsin L. Our study also suggests that the stefin-1 protease inhibitor is released in both species from both NEJ and adult stages (Appendix: Table 7). This stefin stimulates an immune response during the adult stage of infection, as sera from infected rabbits react with recombinant *F. gigantica* serpin at 8 weeks post-infection (Tarasuk et al., 2009). Cystatin-like proteins have also been found in *S. mansoni* and *Haemonchus contortus* (Newlands et al., 2001), the filarial parasite *B. malayi* (Manoury et al., 2001) and in the ESP of the trematode *Paragonimus westermani* (Hwang et al., 2009). Protease inhibitors may have immunomodulatory roles, such as the *Brugia malayi* microfilarial serpin that specifically inhibits proinflammatory serine proteases in human neutrophils (Maizels et al., 2001; Zang et al., 1999). As parasite protease inhibitors have important roles in immunomodulation, these sequences in *F. hepatica* represent an important class of molecules to study further.

3.8 Developmental progression and tegumental proteins

The changes in proteomic profile as the parasites progress from NEJ to adults may be related to the substantial changes in the tegument secretory granules observed as the flukes mature, with NEJ expressing only T0 granules, whereas T1 and T2 granules are expressed in the immature and adult flukes (Bennett and Threadgold, 1975). Moreover, there is a transition from a predominantly secretory function of the intestine in NEJ to absorptive functions in the adult fluke (Bennett and Threadgold, 1973), and there is a drastic change in environment as the NEJ moves from the intestine, to the peritoneum and the liver, then to the bile duct in the adult stage. Rapid switches in antigenic composition of somatic extracts of migrating NEJ have been documented

(Tkalcevic et al., 1996). Direct illustration of this is the presence of peptides matching to a tegumental antigen (AAZ20312) exclusively in adult *F. hepatica* and *F. gigantica* ESP samples and additional sequences from the *F. hepatica* database corresponding to tegumental proteins (ABZ82044 and ABC47326) found exclusively in *F. gigantica* adult ESP.

3.9 Additional proteins

In addition to the stage-specificity of previously characterized enzymes, the relatively high expression of ubiquitin (AAR85354) was common to both *F. gigantica* and *F. hepatica* NEJ (Fig. 16), though it was detected in all samples analyzed. The presence of a ubiquitin pathway was previously described in *Clonorchis sinensis* (Song et al., 2004) and *S. mansoni* (Guerra-Sa et al., 2005). This proteolytic pathway is responsible for the intracellular processes of stage-specific gene transcription and protein quality control and turnover as well as the regulation of membrane-anchored and secretory pathway-compartmentalized proteins (Hochstrasser, 2009). The higher relative abundance of ubiquitin in NEJ may reflect the dynamic changes in protein expression during excystation. In addition to ubiquitin signalling, a 14-3-3 protein (AAW69422) was found to have high relative expression in *F. gigantica* NEJ (Fig. 16), but 14-3-3 sequences were detected in all samples tested (Appendix: Table 7). 14-3-3 proteins are phosphoserine/threonine binding molecules that play a critical role in cell signalling events which control progress through the cell cycle, transcriptional alterations in response to environmental cues and programmed cell death (Yaffe, 2002). A 14-3-3 protein has previously been found in the ESP of *F. gigantica* adults (Chaithirayanon et al., 2006), but its effect on host cells has not been evaluated. The protein may play a role similar to *E. multilocularis* 14-3-3 protein, which reduces mRNA levels of inducible nitric oxide synthetase and nitric oxide production and by host cells activated with LPS *in vitro* (Andrade et al., 2004).

Both actin and tubulin displayed stage- and species-specific expression. Actin (P53471, AAF81130), which has been immunolocalized to tegumental cell bodies of both *F. hepatica* (Stitt et al., 1992b) and *F. gigantica* adults (Tansatit et

al., 2006), represented 4% of the spectra in *F. gigantica* NEJ, 3% in *F. hepatica* adults, and 2% in *F. gigantica* adults (Appendix: Table 7). High expression of fimbrin (AAA29882, AAP06226), a cytoskeletal protein that cross-links F-actin into bundles (Bretscher, 1981), were only detected in *F. gigantica* adults. Beta-tubulin (CAO79607), on the other hand, was found to have high relative expression only in *F. hepatica* adults. Tubulin has been found in the tegumental cell bodies, their cytoplasmic processes and the basal layer of the tegumental syncytium of adult *F. hepatica* (Stitt et al., 1992a) and *F. gigantica* (Tansatit et al., 2006) parasites. Tubulin may be released into the medium as the tegument is sloughed off.

The energy metabolism of the *Fasciola spp.* parasites changes during the development in the host from a predominantly aerobic metabolism in NEJ to a predominantly anaerobic metabolism in adults (Tielens et al., 1982, 1984). As the liver flukes do not have a circulatory system, the availability of oxygen in the tissues depends on diffusion (Tielens et al., 1981a). The rate of aerobic metabolism is therefore proportional to the surface area of the fluke, with a greater surface area relative to body mass in NEJ than adults (Tielens et al., 1982). This change in metabolism is reflected in the relative abundance of oxygen-transport proteins. Myoglobin (AAX11352), sometimes referred to as haemoglobin in parasites, was highly expressed in both *F. hepatica* and *F. gigantica* adults (Fig. 16), whereas hemoglobin F2 (ABW96608) was found exclusively in *F. hepatica* adults (Appendix: Table 7). Myoglobin is involved in O₂ transport and is highly expressed in other trematodes, such as *O. viverrini* (Chutiwitoonchai et al., 2008; Laha et al., 2007), *C. sinensis* (Chung et al., 2003; Ju et al., 2009; Sim et al., 2003), and *P. westermani* (de Guzman et al., 2007). Myoglobin was predicted to be secreted by *F. hepatica* by Robinson et al. (2009), but has not previously been detected in *Fasciola spp.* ESP. *F. hepatica* hemoglobin and its protective effects in vaccination were first reported by Dalton's group (Dalton et al., 1996; McGonigle and Dalton, 1995), and two hemoglobin sequences have been identified in *F. hepatica* (Dewilde et al., 2008), termed hemoglobin F1 and F2. The *F. hepatica* hemoglobin F2 (ABW96608),

identified solely in *F. hepatica* adults in our study, has the conserved Tyr B10/Tyr E7 distal residue pair associated with high oxygen affinity in other trematodes, but the observed oxygen-association rate is lower and the dissociation rate much higher than in other trematodes (Dewilde et al., 2008). In addition, NADP-dependent malic enzyme (CAX72984), an enzyme that is associated with the anaerobic metabolism prevalent in adults, is found exclusively in the adult ESP samples (Appendix: Table 7).

Annexin (CAX70810) was highly expressed in *F. gigantica* adult ESP (Fig. 16), though it was detected in *F. hepatica* adult ESP as well (Appendix: Table 7). Annexins are a family of calcium-dependent phospholipid-binding proteins previously detected in *Schistosoma mansoni* (Braschi et al., 2006), and characterized in *Taenia solium* (Yan et al., 2008) and *Giardia lamblia* (Pathuri et al., 2009). Although annexins lack signal sequences, they occur extracellularly, such as *T. solium* annexin B1 protein (Gao et al., 2007). Annexin B1 has an anti-inflammatory effect *in vitro* (Huang et al., 2006), binds to the extracellular surface of human eosinophils and produces a Ca^{2+} influx that leads to the induction of apoptosis (Yan et al., 2008), and prevents the activation of coagulation factors by binding phosphatidylserine on the surface of activated platelets (Zhang et al., 2007). Annexin proteins in *Fasciola spp.* may have similar effects.

A *C. sinensis* hypothetical protein (AAM55183; Fhep05a12.q1k) was detected only in *F. gigantica* adults (Appendix: Table 7) with high relative abundance (Fig. 16). The hypothetical protein has previously been shown to be an abundant EST in *O. viverrini* (Laha et al., 2007). Its function remains unclear, though it is predicted to have a signal peptide and a transmembrane region.

Enolase (CAK47550) had high relative abundance in all of the samples analyzed (Fig. 16). Enolase has previously been found in adult *F. hepatica* ESP *in vitro* (Jefferies et al., 2001) and *in vivo* (Morphew et al., 2007) and has been found to be specifically recognized by patients in the Peruvian Altiplano, though it was not specific to fasciolosis patients (Marcilla et al., 2008).

Additional adult-specific proteins, including ferritin-like protein (ABC74792), yolk ferritin (AAK35224), and Glyceraldehyde 3-phosphate

dehydrogenase (AAG23287, AAT70328, XP_001478462) (Appendix: Table 7), have recently been identified as antigenic components of soluble egg extracts in *F. hepatica* (Moxon et al., 2010a). Fh-HSP35 α (ACE00520), found exclusively in *F. hepatica* adults (Appendix: Table 7), has recently been identified as a *F. hepatica* egg antigen recognized by sera from infected cattle, rats and goats (Moxon et al., 2010b). With the exception of glyceraldehyde 3-phosphate dehydrogenase, these proteins were detected solely in adult extracts, and may originate from eggs released by the parasites during incubation.

To summarize, comparison of ESP proteomes from *F. hepatica* and *F. gigantica* has revealed greater variability between the NEJ and adult profiles than between the two fasciolid species. Proteases expressed in the NEJ stages were distinct from the adult stages, whereby cathepsin L, cathepsin B and legumain were equally represented in the NEJ and the predominant protease in adult extracts was cathepsin L. The clade-specificity of the peptides detected also grouped according to life stages, with cathepsin L clades A, F and G found exclusively in NEJ and clades B, C, D and E found exclusively in adults. The clade G peptide sequences were detected exclusively in *F. gigantica*. In order to verify *F. gigantica*-specific clades reported in the literature, the specificity of each of the cathepsin L clades should be confirmed by PCR analysis of NEJ and adult cDNA libraries from both species. The clear demarcation of protease profiles between NEJ and adults in both species suggest that proteases with high relative abundance in NEJ, such as cathepsin L clades A and F, cathepsin B clade 3 and NEJ-specific legumain (CAC85636), would be appropriate vaccination or chemotherapeutic targets for early fasciolosis intervention. Other proteins with high relative abundance in NEJ, such as type 3 FABP, should be considered. This study also identified proteins common between both *Fasciola* species and life-stages, including thioredoxin peroxidase and LAP. Thioredoxin peroxidase, which has been shown to play an important role in the immunomodulation of the host immune response (Donnelly et al., 2005), had high relative abundance in all ESP samples analysed and would represent a good chemotherapeutic or vaccine target specific for all stages of fasciolosis. Vaccination with LAP has previously been

shown to provide protection to *F. hepatica* infection in sheep (Piacenza et al., 1999); studies into its effectiveness in *F. gigantica* would be warranted given that the protein was detected in all samples analysed. The relative abundance of certain proteins showed marked differences between the two species, including higher relative abundance of GST enzyme sequence in adult *F. gigantica*. These differences should be confirmed *in vivo* and the functional differences on the survival of the parasite and the effect on the host immune system should be explored further.

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Chapter 4 Appendix

Table 7: Proteins identified in *F. hepatica* and *F. gigantica* NEJ and adult ESP samples by LC-MS/MS

Proteins from *F. hepatica* NEJ, *F. gigantica* NEJ, *F. hepatica* adult and *F. gigantica* adult ESP samples by LC-MS/MS analysis from Trematoda and Mammalia databases as well as proteins identified by searching *F. hepatica* database and BLAST analysis for homologous proteins. Proteins are identified by their Genbank designation. DB indicates the database with which the peptide identification was made (T=Trematoda, M=Mammalia, F=*F. hepatica* EST database). Proteins identified by searching the *F. hepatica* EST database sequences, as indicated by the listing of a *F. h.* reference designation, are listed under their corresponding homologous sequence from BLAST analysis. The number of assigned spectra in each sample is indicated with the number of unique peptides in brackets.

<i>Antioxidant defence enzymes</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Cu/Zn-superoxide dismutase	AAD30361	<i>Fasciola hepatica</i>	T		4 (2)	4 (3)	5 (3)	13 (3)
Cu/Zn-superoxide dismutase	P00442	<i>Bos taurus</i>	M		0 (0)	0 (0)	0 (0)	6 (4)
Glutathione dehydrogenase	CAX73224	<i>Schistosoma japonicum</i>	F	Fhep49e10.q1k	0 (0)	0 (0)	8 (6)	108 (13)
Glutathione transferase (mu class)	ACF59730	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	9 (6)	31 (5)
Glutathione S-Transferase (mu class)	2FHE_A	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	2 (2)	21 (8)
Glutathione s-transferase (sigma class)	ABI79450	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	29 (13)	203 (14)
Glutathione S-transferase (sigma)	AAD23997	<i>Fasciola gigantica</i>	T		0 (0)	0 (0)	0 (0)	6 (2)
Glutathione s-transferase (omega class)	AAO49385	<i>Schistosoma mansoni</i>	F	Fhep49c06.q1k	0 (0)	0 (0)	0 (0)	3 (2)
Glutathione peroxidase	ABK58679	<i>Clonorchis sinensis</i>	F	Fhep45e11.q1k	0 (0)	0 (0)	7 (4)	40 (5)
Thioredoxin	2VIM_A	<i>Fasciola hepatica</i>	T		5 (3)	6 (3)	18 (6)	67 (6)
Thioredoxin-glutathione reductase	CAM96615	<i>Fasciola hepatica</i>	T		0 (0)	3 (3)	4 (4)	37 (13)
Thioredoxin peroxidase	ABY85785	<i>Fasciola gigantica</i>	T		13 (7)	26 (8)	77 (10)	204 (13)

Cell signalling								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Adenylyl cyclase-associated protein	XP_002572870	<i>Schistosoma mansoni</i>	F	Fhep16e11.q1k_p	0 (0)	0 (0)	0 (0)	7 (5)
14-3-3 protein	AAW69422	<i>Fasciola gigantica</i>	T		0 (0)	9 (7)	5 (5)	10 (5)
14-3-3 epsilon	CAZ39172	<i>Schistosoma mansoni</i>	F	Fhep05b09.q1k	2 (2)	5 (5)	2 (2)	11 (5)
14-3-3-like protein	ABL84200	<i>Physarum polycephalum</i>	F	Fhep40g01.q1k	0 (0)	2 (2)	0 (0)	0 (0)
Guanine nucleotide-binding protein Rho	AAY89300	<i>Biomphalaria glabrata</i>	F	Fhep22a09.q1k	0 (0)	2 (2)	0 (0)	0 (0)

Cellular structure and homeostasis								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Actin-2	P53471	<i>Schistosoma mansoni</i>	T		4 (3)	20 (9)	42 (12)	73 (6)
Actin	AAF81130	<i>Idiosepius pygmaeus</i>	F	Fhep54b09.q1k	0 (0)	0 (0)	4 (3)	0 (0)
Alpha-actinin	XP_002572953	<i>Schistosoma mansoni</i>	F	Fhep30a02.q1k	0 (0)	0 (0)	1 (1)	18 (3)
Similar to alpha-actinin	AAP06095	<i>Schistosoma japonicum</i>	T		0 (0)	0 (0)	4 (2)	33 (6)
Carbonyl reductase	XP_002574796	<i>Schistosoma mansoni</i>	F	Fhep43f12.q1k	0 (0)	0 (0)	2 (2)	0 (0)
Putative carbonic anhydrase II	CAX73485	<i>Schistosoma japonicum</i>	F	Fhep18c03.q1k	0 (0)	0 (0)	3 (2)	4 (2)
Decorin precursor	NP_001003228	<i>Canis lupus familiaris</i>	M		0 (0)	0 (0)	8 (4)	0 (0)

<i>Cellular structure and homeostasis</i>								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Putative ezrin	CAX82442	<i>Schistosoma japonicum</i>	F	Fhep54g06.q1k	0 (0)	0 (0)	4 (3)	0 (0)
Fimbrin	AAA29882	<i>Schistosoma mansoni</i>	T		0 (0)	0 (0)	0 (0)	22 (2)
Similar Fimbrin in Schistosoma mansoni	AAP06226	<i>Schistosoma japonicum</i>	F	Fhep32g01.q1k	0 (0)	0 (0)	6 (2)	147 (8)
Heat shock protein 70	ABS52703	<i>Fasciola gigantica</i>	T		7 (5)	5 (5)	34 (18)	55 (18)
Heat shock protein 70	CAM97394	<i>Echinostoma caproni</i>	T		0 (0)	2 (2)	0 (0)	0 (0)
Heat shock protein 90-alpha	P46633	<i>Cricetulus griseus</i>	M		0 (0)	0 (0)	5 (3)	0 (0)
Alpha crystallin-containing small heat shock protein variant NtermFhHSP35a	ACE00520	<i>Fasciola hepatica</i>	F	Fhep22g07.q1k	0 (0)	0 (0)	5 (4)	0 (0)
Histone H4	AAG25601	<i>Schistosoma mansoni</i>	T		5 (3)	0 (0)	0 (0)	0 (0)
Methylmalonyl CoA epimerase	NP_001018602	<i>Danio rerio</i>	F	Fhep33a10.q1k	0 (0)	0 (0)	2 (1)	18 (4)
ornithine aminotransferase	CAX70598	<i>Schistosoma japonicum</i>	F	Fhep50c03.q1k	0 (0)	0 (0)	3 (3)	2 (2)
Peptidylprolyl isomerase A	CAX72371	<i>Schistosoma japonicum</i>	F	Fhep25c09.q1k	7 (5)	13 (7)	31 (9)	116 (8)
Radixin	NP_001009576	<i>Sus scrofa</i>	F	Fhep12d11.q1k	3 (3)	2 (2)	13 (5)	2 (2)
Ribosomal protein rpl40	ABZ04249	<i>Lineus viridis</i>	F	Fhep30g04.q1k	12 (2)	14 (2)	12 (2)	16 (3)
Alpha-tubulin	AAN35134	<i>Entomophaga maimaiga</i>	F	Fhep37d04.q1k	0 (0)	0 (0)	5 (4)	0 (0)
Alpha-tubulin	CAO79606	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	17 (12)	0 (0)
Alpha-tubulin	AAA29918	<i>Schistosoma mansoni</i>	T		0 (0)	0 (0)	2 (2)	0 (0)

Cellular structure and homeostasis								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Alpha-tubulin	CAO79604	<i>Fasciola hepatica</i>	T		3 (3)	0 (0)	0 (0)	0 (0)
Beta-tubulin	CAO79607	<i>Fasciola hepatica</i>	T		2 (2)	2 (2)	151 (20)	9 (6)
Polyubiquitin	BAA09860	<i>Homo sapiens</i>	M		0 (0)	0 (0)	0 (0)	4 (3)
Ubiquitin	AAR85354	<i>Schistosoma mansoni</i>	T		12 (2)	10 (2)	9 (2)	16 (3)
Similar to elongation factor 1 alpha	XP_001254484	<i>Bos taurus</i>	M		0 (0)	0 (0)	3 (2)	0 (0)

Energy metabolism								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Aldolase	CAX71971	<i>Schistosoma japonicum</i>	F	Fhep08d07.q1k	0 (0)	0 (0)	8 (6)	33 (8)
Aldolase	CAX71971	<i>Schistosoma japonicum</i>	F	Fhep13d07.q1k	5 (5)	4 (3)	2 (2)	5 (3)
Aldolase	CAX71973	<i>Schistosoma japonicum</i>	F	Fhep25g04.q1k	2 (2)	0 (0)	3 (2)	0 (0)
Similar to chitinase, acidic	XP_001929072	<i>Sus scrofa</i>	M		0 (0)	20 (8)	0 (0)	0 (0)
Enolase	CAK47550	<i>Fasciola hepatica</i>	T		16 (13)	23 (15)	51 (19)	164 (20)
Enolase	AAN03783	<i>Clonorchis sinensis</i>	F	Fhep05g08.q1k	0 (0)	0 (0)	3 (3)	0 (0)
Similar to alpha-enolase	XP_001362200	<i>Monodelphis domestica</i>	M		0 (0)	0 (0)	0 (0)	2 (2)
Fructose-bisphosphate aldolase	CAC18550	<i>Echinococcus multilocularis</i>	F	Fhep32a03.q1k	0 (0)	0 (0)	10 (7)	0 (0)
Fructose-bisphosphate aldolase isoform A	NP_001164328	<i>Nasonia vitripennis</i>	F	Fhep13a11.q1k	0 (0)	0 (0)	0 (0)	10 (4)

Energy metabolism								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Glyceraldehyde 3-phosphate dehydrogenase	AAG23287	<i>Fasciola hepatica</i>	T		0 (0)	3 (2)	27 (7)	79 (7)
Glyceraldehyde 3-phosphate dehydrogenase	AAT70328	<i>Petromyzon marinus</i>	F	Fhep28h12.q1k	0 (0)	0 (0)	10 (2)	31 (2)
Similar to Glyceraldehyde-3-phosphate-dehydrogeanse	XP_001478462	<i>Mus musculus</i>	M		0 (0)	0 (0)	0 (0)	3 (3)
Lactate dehydrogenase	AAV80238	<i>Clonorchis sinensis</i>	T		0 (0)	1 (1)	5 (3)	0 (0)
Cytosolic phosphoenol carboxykinase	NP_942075	<i>Rattus norvegicus</i>	F	Fhep07g02.q1k	3 (2)	7 (4)	4 (4)	0 (0)
Phosphoenolpyruvate carboxykinase 2	ABZ82032	<i>Clonorchis sinensis</i>	T		1 (1)	0 (0)	4 (3)	0 (0)
Triose-phosphate isomerase	AAZ57433	<i>Orientobilharzia turkestanicum</i>	F	Fhep26g11.q1k	3 (3)	3 (3)	10 (6)	83 (10)
Triosephosphate isomerase	AAC47855	<i>Schistosoma japonicum</i>	T		1 (1)	0 (0)	4 (2)	17 (3)

Inhibition of inflammation								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Annexin	XP_002578945	<i>Schistosoma mansoni</i>	F	Fhep46h08.q1k	0 (0)	2 (2)	0 (0)	48 (9)
Annexin A13	CAX70810	<i>Schistosoma japonicum</i>	F	Fhep46f04.q1k	0 (0)	0 (0)	8 (8)	92 (14)
Similar to Annexin IX CG5730-PC, isoform C	XP_001122440	<i>Apis mellifera</i>	F	Fhep22d12.q1k	0 (0)	0 (0)	0 (0)	7 (2)

Immune system								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Immunoglobulin M heavy chain secretory form	AAN60017	<i>Bos taurus</i>	M		0 (0)	0 (0)	0 (0)	2 (2)

Lipid binding and lipid metabolic process								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Alpha-2-glycoprotein 1, zinc	XP_001143820	<i>Pan troglodytes</i>	M		0 (0)	3 (3)	0 (0)	0 (0)
Fatty acid-binding protein Fh15 (Type 1)	Q7M4G0	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	12 (6)	0 (0)
Fatty acid binding protein (Type 1)	AAB06722	<i>Fasciola gigantica</i>	T		0 (0)	0 (0)	18 (9)	95 (9)
Fatty acid binding-protein type 2	Q7M4G1	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	36 (7)	0 (0)
Fatty acid-binding protein type 3	Q9U1G6	<i>Fasciola hepatica</i>	T		7 (3)	15 (5)	24 (7)	0 (0)
Low density lipoprotein-related protein 12	NP_001128355	<i>Rattus norvegicus</i>	F	Fhep27b08.q1k	5 (2)	0 (0)	7 (3)	25 (2)
Secreted saposin-like protein SAP-3	ABC66278	<i>Fasciola gigantica</i>	T		0 (0)	0 (0)	0 (0)	9 (5)

Proteases								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Calpain 4 6 7 invertebrate	XP_002582147	<i>Schistosoma mansoni</i>	F	Fhep29e12.q1k	0 (0)	0 (0)	11 (8)	0 (0)
Cathepsin B (Clade 1)	Various	<i>Fasciola hepatica</i>	T and F		22 (7)	2 (2)	21 (13)	9 (4)
Cathepsin B (Clade 2)	AAO73003	<i>Fasciola gigantica</i>	T		7 (5)	8 (5)	0 (0)	0 (0)
Cathepsin B (Clade 3)	Various	<i>Fasciola spp.</i>	T		151 (16)	49 (9)	12 (8)	4 (3)

Proteases								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Cathepsin B (Unclassified)	Various	<i>Trichobilharzia szidati</i> and <i>Fasciola hepatica</i>	T and F		0 (0)	0 (0)	13 (10)	4 (4)
Cathepsin L (Clade A)	Various	<i>Fasciola spp.</i>	T		209 (26)	88 (22)	0 (0)	0 (0)
Cathepsin L (Clade B)	Various	<i>Fasciola spp.</i>	T		0 (0)	0 (0)	53 (7)	76 (10)
Cathepsin L (Clade C)	Various	<i>Fasciola spp.</i>	F and T		0 (0)	0 (0)	66 (14)	63 (11)
Cathepsin L (Clade D)	Various	<i>Fasciola spp.</i>	T and F		0 (0)	0 (0)	214 (93)	279 (14)
Cathepsin L (Clade E)	Various	<i>Fasciola spp.</i>	T		0 (0)	0 (0)	14 (6)	3 (2)
Cathepsin L4 (Clade F)	ABZ80400	<i>Fasciola hepatica</i>	T		0 (0)	17 (3)	0 (0)	0 (0)
Cathepsin L6 (Clade G)	ABZ80402	<i>Fasciola hepatica</i>	T		4 (3)	2 (2)	0 (0)	0 (0)
Putative cathepsin-like enzyme (Newly excysted juvenile protein 9)	P80533	<i>Fasciola hepatica</i>	T		0 (0)	8 (2)	0 (0)	0 (0)
Hemoglobinase-like protein 1 (Newly excysted juvenile protein 3)	P80527	<i>Fasciola hepatica</i>	T		7 (1)	0 (0)	0 (0)	0 (0)
Legumain like precursor	CAC85636	<i>Fasciola hepatica</i>	T		236 (15)	55 (11)	0 (0)	0 (0)
Legumain-1	ABQ02437	<i>Fasciola gigantica</i>	T		11 (1)	0 (0)	9 (4)	21 (5)
Legumain-2	ABQ02438	<i>Fasciola gigantica</i>	T		0 (0)	0 (0)	10 (6)	7 (4)
Leucyl aminopeptidase	AAV59016	<i>Fasciola hepatica</i>	T		3 (3)	5 (3)	46 (20)	5 (3)
Putative lysosomal Pro-X carboxypeptidase precursor	CAX71063	<i>Schistosoma japonicum</i>	F	Fhep32e02.q1k	0 (0)	0 (0)	5 (3)	4 (1)
Putative lysosomal Pro-X carboxypeptidase precursor	CAX71065	<i>Schistosoma japonicum</i>	F	Fhep12h05.q1k	0 (0)	0 (0)	7 (4)	0 (0)
Lysosomal pro-X carboxypeptidase	ABJ89817	<i>Clonorchis sinensis</i>	F	Fhep29a07.q1k	0 (0)	0 (0)	3 (3)	22 (4)

<i>Protease inhibitor activity (host)</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Aprotinin analog	1405218A	<i>Bos taurus</i>	M		0 (0)	8 (2)	0 (0)	0 (0)
Similar to alpha-1-antichymotrypsin 2	XP_001928857	<i>Sus scrofa</i>	M		0 (0)	7 (6)	0 (0)	0 (0)
Alpha-1-antichymotrypsin 3	XP_001928637	<i>Sus scrofa</i>	M		0 (0)	2 (2)	0 (0)	0 (0)
Endopin 2B	AAR26722	<i>Bos taurus</i>	M		0 (0)	0 (0)	0 (0)	12 (4)
SERPINA3-2	ABM55496	<i>Bos taurus</i>	M		0 (0)	0 (0)	2 (2)	37 (7)
Similar to SERPINA3-6	XP_001926466	<i>Sus scrofa</i>	M		0 (0)	11 (10)	0 (0)	0 (0)
<i>Protease inhibitor activity (parasite)</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Putative cys1 protein	CAC86126	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	9 (6)	29 (11)
Fh-KTM=6.751 kda monomeric Kunitz-type proteinase inhibitor	AAB46830	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	2 (1)	4 (2)
Stefin-1	ACS35603	<i>Fasciola gigantica</i>	F	Fhep12e07.q1k	4 (2)	6 (2)	5 (2)	36 (3)
Serine protease inhibitor serpin	CAX76360	<i>Schistosoma japonicum</i>	F	Fhep36b11.q1k	0 (0)	0 (0)	0 (0)	5 (2)
Serpin B6 (Placental thrombin inhibitor)	CAX69453	<i>Schistosoma japonicum</i>	F	Fhep20a08.q1k	4 (4)	5 (5)	7 (6)	56 (7)
<i>Tegumental proteins</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Tegumental calcium-binding EF-hand protein	AAZ20312	<i>Fasciola gigantica</i>	T		0 (0)	0 (0)	6 (2)	31 (8)
Tegumental calcium-binding EF-hand protein	ABZ82044	<i>Clonorchis sinensis</i>	F	Fhep49c10.q1k	0 (0)	0 (0)	0 (0)	24 (7)
Tegumental protein 20.8 kDa	ABC47326	<i>Clonorchis sinensis</i>	F	Fhep37g08.q1k	0 (0)	0 (0)	0 (0)	15 (6)

<i>Transport proteins</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Serum albumin precursor	NP_001075813	<i>Oryctolagus cuniculus</i>	M		0 (0)	4 (3)	0 (0)	0 (0)
Ferritin-like protein	ABC74792	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	6 (3)	0 (0)
yolk ferritin	AAK35224	<i>Paragonimus westermani</i>	F	Fhep08c10.q1k	0 (0)	0 (0)	18 (8)	11 (2)
Alpha globin chain	CAA49752	<i>Ovis aries</i>	M		0 (0)	0 (0)	2 (2)	0 (0)
Haptoglobin precursor	NP_001035560	<i>Bos taurus</i>	M		0 (0)	0 (0)	0 (0)	2 (2)
Hemoglobin F2	ABW96608	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	8 (4)	0 (0)
Myoglobin 1	AAX11352	<i>Paragonimus westermani</i>	F	Fhep15f08.q1k	0 (0)	0 (0)	30 (7)	114 (9)
Transferrin	NP_803450	<i>Bos taurus</i>	M		0 (0)	0 (0)	4 (4)	0 (0)
Transthyretin precursor	NP_776392	<i>Bos taurus</i>	M		0 (0)	0 (0)	3 (3)	0 (0)

<i>Proteins with multiple functions and uncharacterized proteins</i>								
Name	Genbank number	Species	DB	<i>F.h.</i> reference	<i>F.h.</i> NEJ	<i>F.g.</i> NEJ	<i>F.h.</i> Adult	<i>F.g.</i> Adult
Calcium-binding protein	CAA06035	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	0 (0)	10 (2)
Calcium-binding protein	CAA06036	<i>Fasciola hepatica</i>	F	Fhep37c02.q1k	0 (0)	0 (0)	5 (2)	43 (8)
Calmodulin-like protein 2	CAL91033	<i>Fasciola hepatica</i>	T		0 (0)	0 (0)	0 (0)	2 (2)
CD63 antigen	CAX72700	<i>Schistosoma japonicum</i>	F	Fhep19h11.q1k	0 (0)	0 (0)	4 (2)	0 (0)
Cytidine deaminase	ZP_04185700	<i>Bacillus cereus</i> <i>AH1271</i>	F	Fhep27h03.q1k	0 (0)	0 (0)	19 (4)	54 (7)
Epididymal secretory protein E1 precursor (Niemann Pick type C2 protein homolog)	CAX71040	<i>Schistosoma japonicum</i>	F	Fhep23e06.q1k	0 (0)	2 (2)	16 (5)	35 (6)

<i>Proteins with multiple functions and uncharacterized proteins</i>								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
Epididymal secretory protein E1 precursor (Niemann Pick type C2 protein homolog)	CAX71037	<i>Schistosoma japonicum</i>	F	Fhep48h03.q1k	0 (0)	0 (0)	4 (2)	3 (2)
Fructose-1,6-bisphosphatase-related	XP_002580627	<i>Schistosoma mansoni</i>	F	Fhep54g11.q1k	0 (0)	0 (0)	2 (1)	7 (3)
Glutamate dehydrogenase	XP_002580330	<i>Schistosoma mansoni</i>	F	Fhep52g05.q1k	0 (0)	0 (0)	3 (3)	0 (0)
Hypothetical protein	XP_001365902	<i>Monodelphis domestica</i>	M		0 (0)	0 (0)	4 (2)	21 (2)
Hypothetical protein	XP_001382006	<i>Monodelphis domestica</i>	M		0 (0)	2 (2)	0 (0)	0 (0)
Hypothetical protein	CAX70856	<i>Schistosoma japonicum</i>	F	Fhep05f07.q1k	0 (0)	0 (0)	5 (4)	8 (5)
Hypothetical protein	CAX69522	<i>Schistosoma japonicum</i>	F	Fhep05f11.q1k	0 (0)	0 (0)	5 (3)	0 (0)
Hypothetical protein	CAX69844	<i>Schistosoma japonicum</i>	F	Fhep13a01.q1k	0 (0)	0 (0)	5 (5)	0 (0)
Hypothetical protein	XP_002579419	<i>Schistosoma mansoni</i>	F	Fhep38d03.q1k	1 (1)	0 (0)	0 (0)	2 (2)
Hypothetical protein	XP_002578722	<i>Schistosoma mansoni</i>	F	Fhep38d06.q1k	0 (0)	0 (0)	0 (0)	5 (3)
Hypothetical protein PANDA_016906	EFB21599	<i>Ailuropoda melanoleuca</i>	F	Fhep10a01.q1k	3 (3)	0 (0)	0 (0)	0 (0)
LAMA-like protein 2 precursor	CAX74468	<i>Schistosoma japonicum</i>	F	Fhep07c04.q1k	0 (0)	0 (0)	2 (2)	2 (2)
Lymphocyte cytosolic protein 1	CAX82387	<i>Schistosoma japonicum</i>	F	Fhep27d03.q1k	2 (2)	2 (1)	16 (4)	141 (11)

<i>Proteins with multiple functions and uncharacterized proteins</i>								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
multidrug resistance protein	XP_002579958	<i>Schistosoma mansoni</i>	F	Fhep08a09.q1k	0 (0)	0 (0)	4 (3)	0 (0)
NADP-dependent malic enzyme	CAX72984	<i>Schistosoma japonicum</i>	F	Fhep27c02.q1k	0 (0)	0 (0)	2 (2)	23 (5)
No significant hits			F	Fhep27d05.q1k	0 (0)	0 (0)	3 (3)	0 (0)
No significant hits			F	Fhep32h03.q1k	0 (0)	0 (0)	3 (2)	0 (0)
No significant hits			F	Fhep40c02.q1k	0 (0)	0 (0)	0 (0)	2 (2)
Putative Fasciola/Schistosoma cross-reactive protein	AAW34056	<i>Fasciola hepatica</i>	F	Fhep37d02.q1k	0 (0)	0 (0)	0 (0)	3 (3)
Putative tyrosine 3-monooxygenase	CAX71274	<i>Schistosoma japonicum</i>	F	Fhep38f04.q1k	0 (0)	0 (0)	0 (0)	4 (3)
Putative uncharacterized protein	AAM55183	<i>Clonorchis sinensis</i>	F	Fhep05a12.q1k	0 (0)	0 (0)	0 (0)	111 (6)
SH3-domain GRB2-like endophilin B1	CAX70483	<i>Schistosoma japonicum</i>	F	Fhep42d11.q1k	0 (0)	1 (1)	0 (0)	5 (4)
Similar to Cubulin precursor	XP_002129158	<i>Ciona intestinalis</i>	F	Fhep11a12.q1k	0 (0)	2 (2)	5 (4)	21 (2)
Similar to Hist1h4c protein	XP_604589	<i>Bos taurus</i>	M		2 (2)	0 (0)	0 (0)	0 (0)
Similar to pepsinogen C	XP_001928940	<i>Sus scrofa</i>	M		0 (0)	3 (2)	0 (0)	0 (0)
SJCHGC00820 protein	AAW27659	<i>Schistosoma japonicum</i>	T		0 (0)	3 (3)	9 (4)	0 (0)
SJCHGC00969 protein	AAW27532	<i>Schistosoma japonicum</i>	F	Fhep23d12.q1k	0 (0)	0 (0)	3 (3)	4 (2)
SJCHGC01661 protein	AAW25384	<i>Schistosoma japonicum</i>	F	Fhep18a08.q1k	0 (0)	0 (0)	3 (2)	2 (1)
SJCHGC06124 protein	AAW27425	<i>Schistosoma japonicum</i>	T		0 (0)	0 (0)	2 (2)	0 (0)

<i>Proteins with multiple functions and uncharacterized proteins</i>								
Name	Genbank number	Species	DB	F.h. reference	F.h. NEJ	F.g. NEJ	F.h. Adult	F.g. Adult
SJCHGC06318 protein	AAW25537	<i>Schistosoma japonicum</i>	T		0 (0)	8 (2)	14 (4)	60 (5)
SJCHGC06332 protein	AAW25470	<i>Schistosoma japonicum</i>	F	Fhep28c04.q1k	0 (0)	0 (0)	4 (3)	0 (0)
SJCHGC09432 protein	AAW24824	<i>Schistosoma japonicum</i>	T		0 (0)	0 (0)	2 (2)	2 (2)
SJCHGC09717 protein	AAX31013	<i>Schistosoma japonicum</i>	F	Fhep45h08.q1k	0 (0)	0 (0)	0 (0)	5 (2)
Sphingomyelin phosphodiesterase	ABL07370	<i>Clonorchis sinensis</i>	F	Fhep16a11.q1k	0 (0)	0 (0)	17 (5)	5 (4)
T-cell-stimulating antigen	CAA67208	<i>Schistosoma mansoni</i>	F	Fhep25a07.q1k	0 (0)	0 (0)	0 (0)	9 (2)
Translation initiation inhibitor	XP_002576820	<i>Schistosoma mansoni</i>	F	Fhep06b08.q1k	0 (0)	0 (0)	4 (3)	0 (0)
Unknown protein	AAI40643	<i>Bos taurus</i>	M		0 (0)	0 (0)	0 (0)	8 (3)
Unknown protein	AAW24979	<i>Schistosoma japonicum</i>	T		0 (0)	1 (1)	4 (2)	0 (0)
Unnamed protein product	BAE27157	<i>Mus musculus</i>	F	Fhep38d08.q1k	3 (2)	0 (0)	7 (3)	0 (0)
UVS.2 protein	AAA49980	<i>Xenopus laevis</i>	F	Fhep40f07.q1k	0 (0)	0 (0)	12 (5)	35 (5)

Connecting statement 3

The following Chapter is an analysis of the protein profiles of cattle infected with *F. hepatica* during the first 14 weeks of infection. This work complements the analysis carried out in Chapter 2 by looking at the response of cattle that express a degree of resistance to reinfection by the parasite. Samples analysed in this study were well-characterised sera obtained from animals in a vaccine trial. The treatment group had been administered a proprietary antigen with adjuvant prior to infection and the control group received the adjuvant alone. Both groups were infected in a trickle infection rather than a single-dose infection, in contrast to the study in the second Chapter. Trickle infections are a better simulation of the type of infection to which animals are exposed in the field. The goals of this study were to identify markers of responsiveness to vaccination in the immunized group and markers of infection in the control group. The research methods to be used in this analysis were determined prior to knowing the outcome of the vaccine trial, which showed that exposure to the vaccine antigen did not confer protection, as seen in Fig. 21. The analysis of sera from immunised animals did not show markers of responsiveness by SELDI-TOF MS analysis. The following Chapter describes the profiling of proteins in sera of *F. hepatica* trickle-infected control cattle. The methodology in this Chapter was modified from the methodology in the second Chapter in order to address shortcomings in the previous analysis.

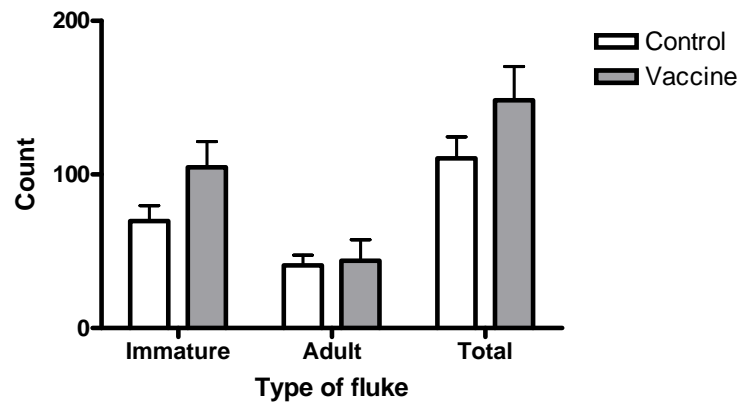


Figure 21: Parasite count at the end of the vaccine trial, including immature and adult parasites. The data indicate that there was no significant difference between the control group and the treatment group exposed to antigen.

Chapter 5:

Analysis of the serum proteome of *F. hepatica* trickle-infected cattle

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Abstract

Serum biomarkers associated with *Fasciola hepatica* infection in trickle-infected cattle were analysed during the first fourteen weeks of infection using SELDI-TOF Mass Spectrometry (MS) as well as one-dimensional gel electrophoresis combined with tandem MS of immunodepleted sera. Two markers of infection were identified by SELDI-TOF MS: a marker at 1852 Da was significantly decreased at 6 and 10 weeks post-infection (w.p.i.), corresponding to the immature/mature stage of infection, and a marker at 8364 Da that was significantly decreased at 3 and 6 w.p.i., during the acute stage of infection, and significantly increased at week 14 p.i., during the chronic stage of infection. Immunoaffinity depletion of the sera from infected cattle increased the relative abundance of low abundance proteins in cattle sera and permitted the identification of markers for the acute phase response, liver fibrosis and immunomodulation by the parasite, although it was not sufficiently sensitive to identify parasite proteins. The study of the host response to a trickle-infection may provide a more accurate representation of the natural pathogenesis of *F. hepatica* and the development of immunity in the field and help to expand our understanding of the immune response that can develop to *F. hepatica* in cattle.

Keywords: *Fasciola hepatica*, SELDI-TOF MS, biomarkers, serum, immunoaffinity depletion, trickle infection

1. Introduction

Fasciola hepatica is an important veterinary parasite of ruminants, including sheep, cattle, and buffalo (McManus and Dalton, 2006). Veterinary fasciolosis is estimated to cause losses of 3.2B USD (Spithill et al., 1999). Although lethal outbreaks are uncommon among cattle, modest fluke infections can result in significant reductions in performance, including weight gain, fertility and milk production. Studies have estimated losses of 0.7 kg of milk per infected cow per day (Charlier et al., 2007), with an overall loss of €300 per infected animal (Schweizer et al., 2005). Globally, infections in cattle are widespread, with prevalence rates in cattle estimated at 18% in Switzerland (Rapsch et al., 2006), 39-78% in Vietnam (Anderson et al., 1999; Geurden et al., 2008), and 48% in England and Wales (Salimi-Bejestani et al., 2005).

An animal becomes infected with *F. hepatica* after ingesting water or food contaminated with encysted metacercariae. The metacercariae excyst in the small intestine within a few hours of ingestion. Once excysted, the parasites migrate through the mucosa and epithelia of the small intestine and through the peritoneum. The juvenile fluke penetrates the liver capsule within a few days and causes inflammation as the growing parasite feeds on the liver parenchyma (Andrews, 1999). This acute parenchymal phase of the disease is characterized by anemia due to the mechanical damage caused by the spines and oral sucker of the parasite, as well as the direct blood loss from the parasite feeding at the rate of 0.2-0.5mL of blood per day per fluke (Dawes and Hughes, 1964). A cellular inflammatory reaction to the mechanical damage in the liver occurs (Dow et al., 1967, 1968; Ross, 1967) and fibrosis results in cattle (Ross et al., 1966). Glutamate dehydrogenase (GLDH) (E.C. 1.4.1.2) activity in the serum is used as a marker of parenchymal tissue damage in this phase of the infection. Increased GLDH levels are seen as early as two weeks after infection in calves (Anderson et al., 1977) with maximal levels coinciding with the end of the parenchymal phase, about 13 weeks post-infection (w.p.i.) (Anderson et al., 1981; Sykes et al., 1980). The parasite enters the bile duct as it reaches maturity, approximately seven to ten

weeks after infection, reaching patency (as defined by egg shedding) between eight and ten weeks post-infection. This chronic stage of the disease is characterized by bile duct damage and increased γ -glutamyl transferase (GGT) (E.C. 2.3.2.2) activity in serum, while the inflammation of the parenchymal tissues subsides (Bulgin et al., 1984). Chronic disease can persist for several years in cattle.

Cattle, unlike sheep, are capable of expelling an initial infection with *F. hepatica* and can also resist reinfection upon secondary exposure to the parasite, as measured by a decrease in the size and number of flukes recovered from the challenge. In early studies, this resistance was correlated with the duration of primary infection (Doyle, 1971, 1973), and is believed to be due at least in part to the hepatic fibrosis and calcification of the bile ducts that results from the migration and feeding of the parasite during infection (Boray, 1967; Ross, 1967). Recent studies suggest that protective histological changes resulting from infection require large single challenge doses (>500 metacercariae); resistance is not appreciable in trickle challenges simulating field infections by administration of 50 metacercariae per day for 10 days. There is evidence that a protective immune response can be mounted against *F. hepatica* infections following vaccinations or drug abbreviated infections (McManus and Dalton, 2006). Hoyle et al. (2003) measured the effects of drug-abbreviated infections on reinfection in two ways: (i) by exposing animals to the early migration of newly excysted juveniles across the gut into the peritoneal cavity with exposure to three 1-day infections, (ii) allowing the migration of juvenile flukes into the peritoneal cavity and into the liver with exposure to a 5-day infection. The study demonstrated that 5-day or three 1-day abbreviated infections significantly reduced liver damage following secondary challenge, as measured by glutamate dehydrogenase level in serum, γ -glutamyltransferase activity and lower eosinophilia. This suggests that exposure to antigens released by migrating juvenile flukes, without damage to the liver or bile ducts, is protective.

The immune response to *F. hepatica* infection in cattle is characterized by elevated eosinophilia at 4 weeks post-infection that persists into the chronic stage

of infection (Bossaert et al., 2000b) and by stimulation of the alternative activation pathway of macrophages both *in vivo* and *in vitro* (Flynn et al., 2007). Alternatively activated macrophages are characterized by high levels of arginase activity, and have been associated with two principal functions: stimulation of T_H2 cell differentiation by stimulating T_H2 cytokine secretion from CD3 stimulated naïve cells, as reported in murine model and the suppression of T_H1 inflammatory responses (Donnelly et al., 2008). In mice, *F. hepatica* infection induces the alternative activation of dendritic cells. Dendritic cells exposed to excretory-secretory products (ESP) from *F. hepatica* primed CD4⁺ cells to produce IL-4, IL-5 (type 2 cytokines), and more than 3 times the amount of IL-10 and TGF- β regulatory cytokines, as compared to cells primed with immature DC, while at the same time reducing the production of IFN- γ induced by LPS (Falcon et al., 2010). Stimulation of dendritic cells with *F. hepatica* tegument antigen has been shown to downregulate the activation markers CD40, CD86 and CD80 as well as IL-12p70, IL-6, TNF- α and nitrite production of LPS-stimulated cells in a TLR-4-independent fashion in mice (Hamilton et al., 2009).

Early activation of antigen presenting cells may be critical for the polarization of the immune response early in infection from an early T_H1/T_H0 response to a T_H2 response as the infection becomes established (Flynn and Mulcahy, 2008b). There is an early production of IFN- γ , with maximal production by peripheral blood lymphocytes (PBL) in response to *F. hepatica* antigens found at 2-5 w.p.i. in cattle (Clery and Mulcahy, 1998), with no significant IFN- γ production at eight weeks post-infection (Clery et al., 1996). IL-4 levels rise early in infection (Waldvogel et al., 2004) to peak levels in PBMC 4-6 w.p.i. (Flynn and Mulcahy, 2008a, b). IL-4 has a strong influence on the differentiation of naïve T_H cells into T_H2 cells and may inhibit their differentiation into T_H1 cells (Waldvogel et al., 2004). Levels of IL-10 produced *in vitro* by PBMCs from *F. hepatica*-infected cattle increase and remain elevated over the course of infection (Flynn and Mulcahy, 2008a), which has been associated with a suppression of IFN- γ production in infected cattle *in vitro* (Flynn and Mulcahy, 2008b). The parasite-specific antibody isotype profile over the course of infection

mirrors the cytokine profiles, with predominantly IgG1 antibodies (Brown et al., 1999; Clery et al., 1996; Spithill et al., 1997) appearing and peaking within 4 w.p.i. (Bossaert et al., 2000a; Clery et al., 1996). The IgG1 response induced in experimental *F. hepatica* infection is not protective, in that IgG1 titres are either not correlated with parasite burden (Bossaert et al., 2000a), or show a positive correlation (Mulcahy et al., 1998). Clery et al. (1996) demonstrated a positive correlation between PBL responsiveness to fluke antigen and adult fluke burden, suggesting that animals with the strongest fluke antigen-induced lymphocyte responses were the least able to expel the adult flukes.

In addition to immunomodulation towards a T_H2 response, *F. hepatica* antigens can also suppress immune responses. Cathepsin L1 and glutathione *S*-transferase σ induce suppressive effects in murine dendritic cells (Dowling et al., 2010). The cells are capable of suppressing a T_H17 response but fail to activate a T_H2 response. Donnelly et al. (2010) demonstrated reduced macrophage recognition of TLR-4 and TLR-3 ligands as a result of TLR-3 degradation by cathepsin L1 in the endosome. *F. hepatica* ES and recombinant cathepsin L can cleave CD4 and can suppress the Con A-stimulated proliferation of lymphocytes from naïve sheep *in vitro* (Prowse et al., 2002).

Profiling the serum proteins of *F. hepatica*-infected cattle at different time points provides a snapshot of the host-parasite interplay and can highlight biomarkers, or key indicators of the infection or the response to infection. Biomarkers include indicators of early parasite migration, response to juvenile parasites, the acute phase response or chronic infection. Serum analysis has been used to identify biomarkers of *F. hepatica* infection in sheep (Rioux et al., 2008) as well as Chagas' disease in humans (Ndao et al., 2010b). In this study, we describe the use of SELDI-TOF MS to generate proteomic profiles of sera from *F. hepatica*-infected cattle for the first 14 weeks of infection. In addition, we describe the use of immunoaffinity depletion using an IgY-12 column which depletes the 12 most abundant serum proteins and its use in the comparative profiling of serum proteins of uninfected cattle, cattle with acute *F. hepatica* infections and chronic *F. hepatica* infections using 1DE and LC-MS/MS.

Depletion of abundant proteins was used to increase the likelihood of identifying markers of infection in the sera of *F. hepatica*-infected cattle. Such protein profiles could provide insight into the mechanisms of resistance of cattle to *F. hepatica* infection and identify early indicators of bovine infection.

2. Materials and Methods

2.1 Cattle serum samples

Serum samples were obtained from Pfizer Animal Health, Sandwich, U.K. The samples were obtained from control group animals in a vaccination trial. Each animal was orally challenged with a gelatin capsule containing 40 metacercariae per day, three times per week for six weeks and humanely slaughtered at week 14 post-infection. Flukes were removed from the liver and gall bladder after slaughter. Adults were defined as flukes that were recovered from the major bile ducts and gall bladder; immature flukes were defined as those that were removed from the minor bile ducts and the liver parenchyma.

The average number of flukes recovered in the control group was 111, with approximately 70 immature and 41 adult parasites. Blood was collected from all cattle prior to infection and then weekly until the time of slaughter. The sera were stored at -80°C.

2.2 Serum fractionation

Sera were fractionated using a Ciphergen Q Hyper D F strong anion-exchange resin filtration plate. The filtration plate was re-equilibrated by adding 200 µl rehydration buffer (50 mM Tris-HCl, pH 9.0) and placed on a Micro-Mix 5 orbital vortex (Beckman Coulter) (form 20 and amplitude 7) for 60 min at room temperature (RT). The rehydration buffer was removed by vacuum and the resin was washed four times with 200 µl rehydration buffer and four times with 200 µl U1 solution (1 M urea, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 9.0). Serum samples were thawed on ice and centrifuged at high speed (17,300 g) for 5 min at RT to remove particulates. Twenty µl sample aliquots were added to a v-bottom 96-well

microplate (Costar Corning) with 30 μ l U9 buffer (9 M urea, 2% CHAPS, 50 mM Tris-HCl, pH 9). The microplate was sealed and placed on a MicroMix 5 orbital vortex (form 20, amplitude 5) for 20 min at RT. Fifty μ l samples were added to the equilibrated HyperD F resin with 50 μ l U1 buffer. The filtration plate was sealed and placed on the MicroMix 5 orbital vortex (form 20, amplitude 7) for 30 min at RT and fractions were collected by vacuum. One hundred μ l pH 9 buffer [50 mM Tris-HCl, 0.1% octyl b-D-glucopyranoside (OGP), pH 9] were added to the wells of the filtration plate using the Biomek robot automation system (Beckman Coulter), the microplate was placed on the MicroMix 5 orbital vortex at RT for 10 min and the fraction collected by vacuum. One hundred μ l of the following buffers were added in two consecutive applications and collected by vacuum: pH 9 buffer, pH 7 buffer (50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.1% OGP, pH 7), pH 5 buffer (100 mM sodium acetate, 0.1% OGP, pH 5), pH 4 buffer (100 mM sodium acetate, 0.1% OGP, pH 4), pH 3 buffer (50 mM sodium citrate, 0.1% OGP, pH 3), and organic wash buffer (33.3% isopropanol, 16.7% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA)). The two 100 μ l eluants from each pH fraction were pooled, aliquoted and stored at -80°C.

2.3 Binding of fractions to ProteinChip[®] arrays

Binding of fractions was performed similarly to methods previously described (Rioux et al., 2008). Fractions of serum were profiled on a weak cation-exchange (CM10) ProteinChip[®] Array according to the manufacturer's instructions. All steps were performed at RT. Briefly, the ProteinChip[®] arrays were placed in a Ciphergen bioprocessor (C503-0006) and washed twice with 200 μ l low-stringency binding buffer (0.1 M sodium acetate, pH 4) and placed on a multitube vortexer (VWR VX-2500) at speed 1 for 5 min. Each fraction was bound to the chip by adding 20 μ l sample in 180 μ l binding buffer; the bioprocessor was placed on the multi-tube vortexer for 60 min. The samples were discarded, the ProteinChip[®] arrays washed three times with 200 μ l binding buffer, placed on the multitube vortexer for 5 min and washed twice with 200 μ l ddH₂O

for 1 min. The ProteinChip[®] arrays were air-dried prior to matrix application. All samples were randomized using Ciphergen Express Software and loaded accordingly.

The proteins from cattle serum depletion studies were bound non-specifically to a gold-coated ProteinChip[®] array (Ciphergen Biosystems) by applying 1 µl sample. The samples were analyzed as described below.

2.4 Preparation and application of matrix

A saturated sinapinic acid (SPA) solution was prepared by adding 50% ACN/0.5% TFA solution to 5 mg of SPA and placed on a tube vortex (Fisher 12-812) at a high setting for 5 min, left to stand for 5 min and centrifuged for 10 min at 17 300g. Matrix (1 µl) was added to each spot on the ProteinChip[®] array and air-dried prior to adding an additional 1 µl of matrix.

2.5 SELDI-TOF MS analysis

ProteinChip[®] arrays were read using the Ciphergen PSC 4000-1213 SELDI-TOF MS reader. Profiles were collected in two separate ranges: 0-100 kDa, with a focus mass of 3.7 kDa (low energy, or LE, protocol), and 0-200 kDa, with a focus mass of 19 kDa (high energy, or HE protocol). The laser intensity was optimized during each data collection. The LE protocol had a matrix attenuation of 500 Da, a sampling rate of 800 MHz, and one warming shot at the collection intensity. The HE protocol had a matrix attenuation of 5000 Da, a sampling rate of 400 MHz, and a warming shot at the collection intensity. Spectra were calibrated using all-in-one protein standard (Ciphergen Biosystems) using the following standards for spectra collected with the LE protocol: [arg⁸]-vasopressin, somatostatin, dynorphin A (porcine), ACTH [1-24] (human), insulin β-chain (bovine), insulin (human recombinant), hirudin (recombinant); and the following proteins for the spectra collected using the HE protocol: cytochrome c (bovine), myoglobin (equine cardiac), carbonic anhydrase (bovine RBC), enolase (*S. cerevisiae*), albumin (bovine serum), IgG (bovine).

2.6 CIPHERGEN EXPRESS SOFTWARE ANALYSIS

Spectra were analyzed using Ciphergen Express 3.0.6 software. External calibrations from appropriate standards, collected on the same date and using the same protocol as the samples, were applied to the spectra. The baseline was adjusted to fit 5 times the expected peak width. The spectra were normalized from a minimum mass-to-charge (M/z) of 1500 Da to a maximum of 100 000 Da in LE protocols and from 5000 Da to 200 000 Da in HE protocols. Peaks were automatically detected using 6.0 times noise, within a mass window of 1.5-100 kDa in LE protocols and 5-200 kDa in HE protocols. The centroid fraction was set to 10% and all peaks were cleared prior to detection. The week with a normalization factor closest to 1.0 was used as the reference spectrum to align spectra to each other. Default conditions were used to match peaks (0.3% mass window, signal-to-noise ratio (S/N) less than 5.0). The spectra alignment was only accepted if the percentage coefficient of variation (CV) was reduced. Peak detection and clustering were automated. Peaks were automatically detected on the first pass when the S/N was 5, and the peak was five times the valley depth. User-detected peaks did not override these minimal criteria. Clusters were created within 0.3% of M/z for each peak detected in the first pass. The clusters were completed by adding peaks with a S/N of 2 and two times valley depth. When no peaks were detected, the peak intensity was estimated at the centre of the cluster.

In the discovery study, peaks were screened for potential biomarkers using the following criteria: marker is up-regulated or down-regulated two-fold in two or more weeks post-infection in two independent fractionations and binding analyses. In order to compute these differences, the numerical data for each cluster were exported as .csv files from Ciphergen Express software program and analysed using MATLAB. Experimental week data were compared to the uninfected control and 2-fold or greater differences (ratios of >2 for up-regulated markers, <0.5 for down-regulated markers) were retained. Markers with at least two data points retained in both analyses with consistent patterns of up- or down-regulation were retained. The peaks were inspected to ensure that none were

multi-charged entities. Fractions that contained markers of interest were retained for the validation study.

For the validation study, p-values and receiver operation characteristic (ROC) values were calculated using CIPHERGEN Express Software by comparing control infection groups, one week at a time using a paired non-parametric *t*-test. *P*-values < 0.05 were considered statistically significant.

2.7 Depletion of high abundance serum proteins using IgY-12 column

High-abundant proteins were depleted using a 6.4 x 63 mm IgY-12 High Capacity LC2 column (Beckman Coulter Inc., Brea, CA) according to manufacturer's protocols. The column contains affinity-purified IgY antibodies specific for human albumin, IgG, fibrinogen, transferrin, IgA, IgM, HDL (including Apo A-I and apo A-II), haptoglobin, α 1-antitrypsin, α 1-acid glycoprotein, and α 2-macroglobulin. Immunoaffinity chromatography was conducted on a Beckman Coulter HPLC (Beckman Coulter Inc., Brea, CA). Prior to injection on the column, serum samples were diluted four times with Buffer A (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing one tablet of mini protease inhibitor (Roche) per 10 ml (112.5 μ l sample, 337.5 μ l dilution buffer). The sample was spun in a 0.45 μ m spin filter at 9 200 *g* for 1 min at 4°C. One hundred μ l of sample was injected onto IgY-12 LC2 column in 100% Buffer A at a flow rate of 0.1 ml/min for 10 min, 0.2 ml/min for 7 min, and 1.0 ml/min for 5 min. After collection of the flow-through fraction, the remaining proteins were eluted with 100% Buffer B (0.1M Glycine-HCl, pH 2.5) at a flow rate of 1.0 ml/min for 19 min. Afterwards, the column was neutralized with Buffer C (0.1 M Tris-HCl, pH 8.0) at a flow rate of 1.0 ml/min for 6 min and reequilibrated with Buffer A for 6 min for a total run cycle of 53 min. Elution of proteins was monitored at 260 nm and 280 nm, as described (Yocum et al., 2005) and collected accordingly. Low-abundance serum proteins were collected manually from 10-19 min and high abundance proteins from 33-40 min. The high abundance fraction collected was neutralized with Buffer C. Samples were concentrated

using 3 kDa molecular weight cutoff (MWCO) centrifugal filters (Millipore, Billerica, MA) at 4000 g for 45 min at 4°C. Protein concentration was quantified using the Qubit fluorometer (Invitrogen, Carlsbad, CA) and samples stored at -80°C.

Individual samples from 0 w.p.i. (control serum), 1-7 w.p.i. (juvenile and immature infection stages), and 13-14 w.p.i. (adult stage) were pooled. Twelve depletions for each set of pooled samples were completed prior to proceeding with LC-MS/MS analysis.

2.8 1DE of depleted proteins

Ten µl of low abundance proteins and high abundance proteins eluted from three independent depletion runs of cattle serum, equivalent to approximately 500 ng protein, were analyzed on a 4-12% Bis-Tris NuPAGE gel using MOPS running buffer under reducing conditions (Invitrogen) according to the manufacturer's instructions. The gels were stained with Silver Stain Plus (Invitrogen). A 0.1 µl aliquot of serum was analyzed alongside the depleted sera.

2.9 Sample preparation and LC-MS/MS analysis

After concentration using 3 kDa MWCO centrifugal filters, the entire volume of the retentate was concentrated using TCA precipitation. The proteins were separated using a 4-15% Bis-Tris gel and the gel was sliced into 10 separate fractions and each slice was subjected to trypsin digestion and LC-MS/MS analysis. The resulting peptides were identified using a Mammalia/Trematoda database. Proteins with two or more unique peptides were retained. Proteins with ≥ 3 -fold differences in peptide count were considered markers of infection.

3. Results

3.1 Infection

The trickle infections were successful, with a median worm burden of 111 (standard deviation of 41.7), of which 70 were immature and 41 were adult parasites (Table 8). *F. hepatica* parasites usually reach the bile ducts 7-10 w.p.i. (Andrews, 1999), and reach patency 8-10 w.p.i. The first seven weeks of infection should be characterized by the presence of juvenile and immature parasites, with an acute phase (Fig. 22). By 8 w.p.i., a gradual decline and a concomitant increase in adult parasites is expected until all parasites reach maturity, which should occur between 14-16 w.p.i. Post-mortem analysis of the location of the parasites at week 14 indicated that the parasites were not all mature, and that the infection was in the late acute and early chronic stage. The mixed population of parasites (Fig. 22) best simulated the type of infection seen in the field, where animals are consistently consuming low doses of metacercariae, but it does complicate interpretation of the stage-specificity of the markers of infection.

Cow number	Immature flukes	Adult flukes
1253	93	54
4427	42	31
3738	72	44
1190	102	19
2191	55	35
2319	123	74
5147	43	31
4154	44	64
2524	53	15

Table 8: Immature and adult fluke parasite counts, listed by experimental animal.

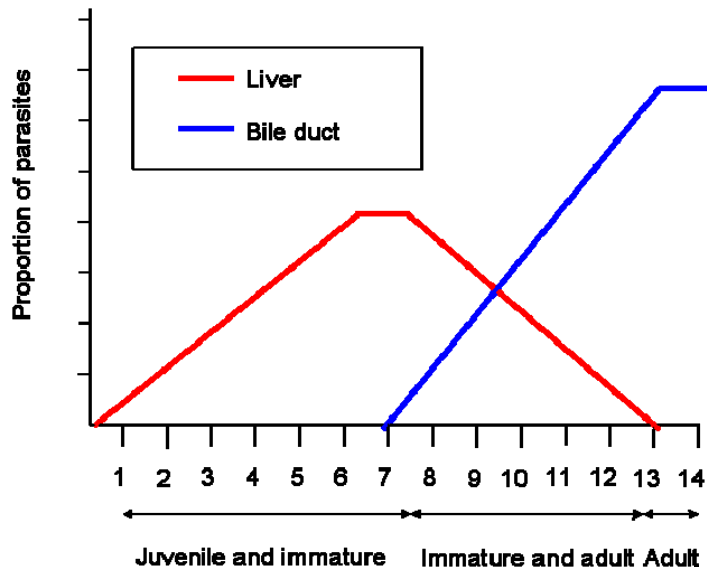


Figure 22: *F. hepatica* maturation in cattle in a trickle-infection model

Representation of the parasites expected during the course of *F. hepatica* infection in cattle given a dosing schedule of 40 metacercariae per day, three times per week for six weeks.

3.2 SELDI-TOF MS analysis

As seen in Fig. 23, the spectrum from the pH9 fraction of pooled sera from 6 w.p.i. is distinct from the pH9 fraction of pooled control sera. Analysis of the clustering data from the SELDI-TOF MS spectra identified 23 peptides (1.5-10 kDa) and 27 proteins (10-200 kDa) as candidate markers in the pH 9 and organic wash fractions (Table 9). Of these markers, 27 were down-regulated and 23 were up-regulated. Weeks 0 (control), 2, 3, 4, 6, 10 and 14 p.i. were used for the individual fractionation analysis to cover the juvenile-immature stages (2, 3, 4, and 6 w.p.i.), immature-adult (10 w.p.i.) and adult (14 w.p.i.) stages.

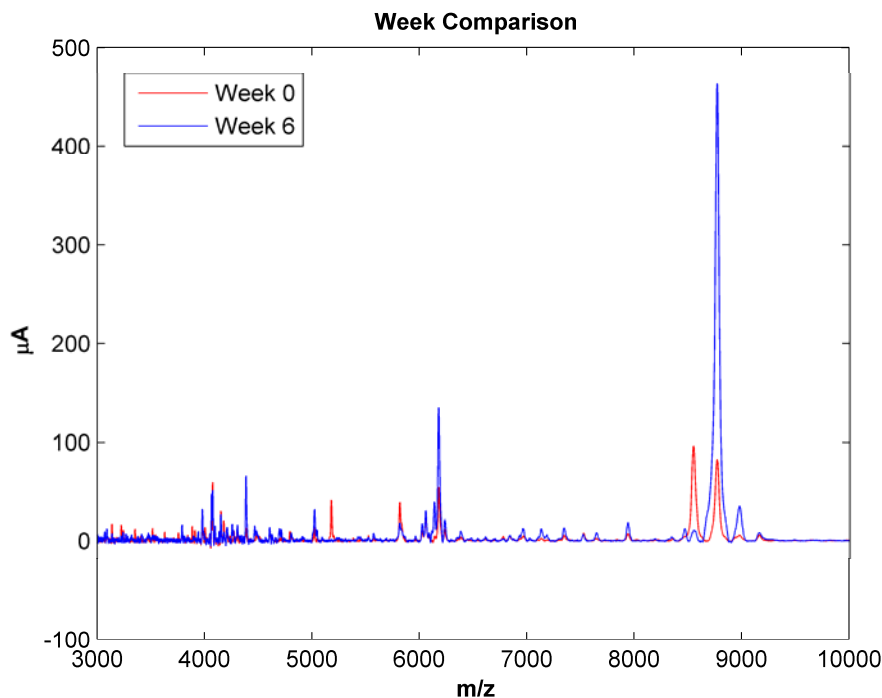


Figure 23: Sample spectra from preliminary SELDI-TOF MS marker study

Comparison of spectra at week 0 post-infection (red) and week 6 post-infection (blue) shows differences in peak intensity at specific m/z on MS output. The intensities of the peaks were compared in MATLAB to identify a list of candidate markers to be studied in the validation study.

Table 9: Potential markers identified in preliminary SELDI-TF MS analysis of fractions of pooled cattle serum

Number of potential markers in pH9 and organic wash fractions where peak intensities showed 2-fold decreases (↓) or increases (↑) during infection as compared to week 0 p.i. reference spectrum in at least two weeks post-infection

Fraction	Range	↓	↑
pH9	1.5-10 kDa	9	3
	10-200 kDa	6	5
Organic wash	1.5-10 kDa	8	3
	10-200 kDa	4	12
Total		27	23

Of fifty potential markers identified in the preliminary study, two were found to be significantly ($P < 0.05$) increased and/or decreased: 1852 Da in the pH 9 fraction and 8364 Da in the organic wash fraction. The 1852 Da marker from the pH 9 fraction was decreased around 10 w.p.i. and significantly decreased at 6 and 10 w.p.i., corresponding to the acute liver phase of infection. The

8364 Da marker from the organic wash fraction was consistently decreased in intensity between 6-9 w.p.i., and increased at 14 w.p.i. in the preliminary study (Fig. 24). The 8364 Da trace was significantly decreased at 3 and 6 w.p.i., and significantly increased at 14 w.p.i., in the validation study (Fig. 25). The decrease in intensity of this peak at 6-9 w.p.i. corresponds to the acute liver stage of the disease, whereas the significant increase at 14 w.p.i. coincides with the maturation of the parasite in the bile duct and the chronic stage of the disease.

Purification of the 1852 Da and 8364 Da markers was attempted by bulk anion-exchange fractionation of pooled samples from 0, 3, 6, 10 and 14 w.p.i. followed by hydrophobic fractionation of the pH 9 and organic wash fractions, but the markers were not present in sufficient abundance for identification by LC-MS/MS.

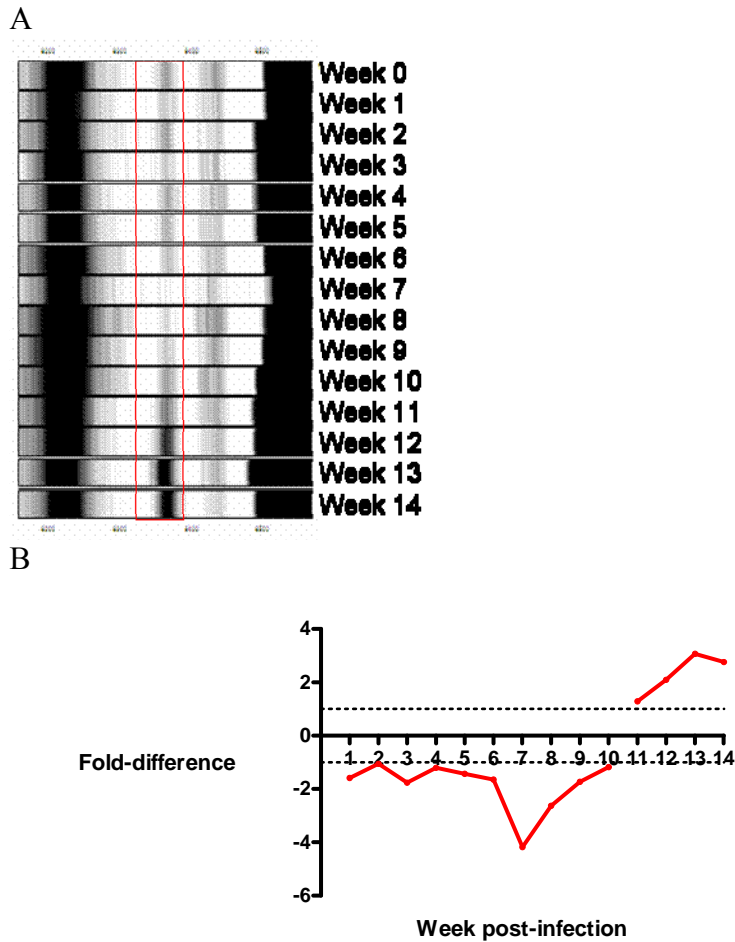


Figure 24: Marker of *F. hepatica* trickle infection identified in preliminary SELDI-TOF MS study

A trace at 8364 Da was decreased at 4- 9 w.p.i. and increased at 13-14 w.p.i. in a preliminary study in the organic wash fraction from pooled cattle samples, as seen in the gel-view image of a representative set of SELDI-TOF MS spectra of pooled sera (A) and fold-difference from control values (B).

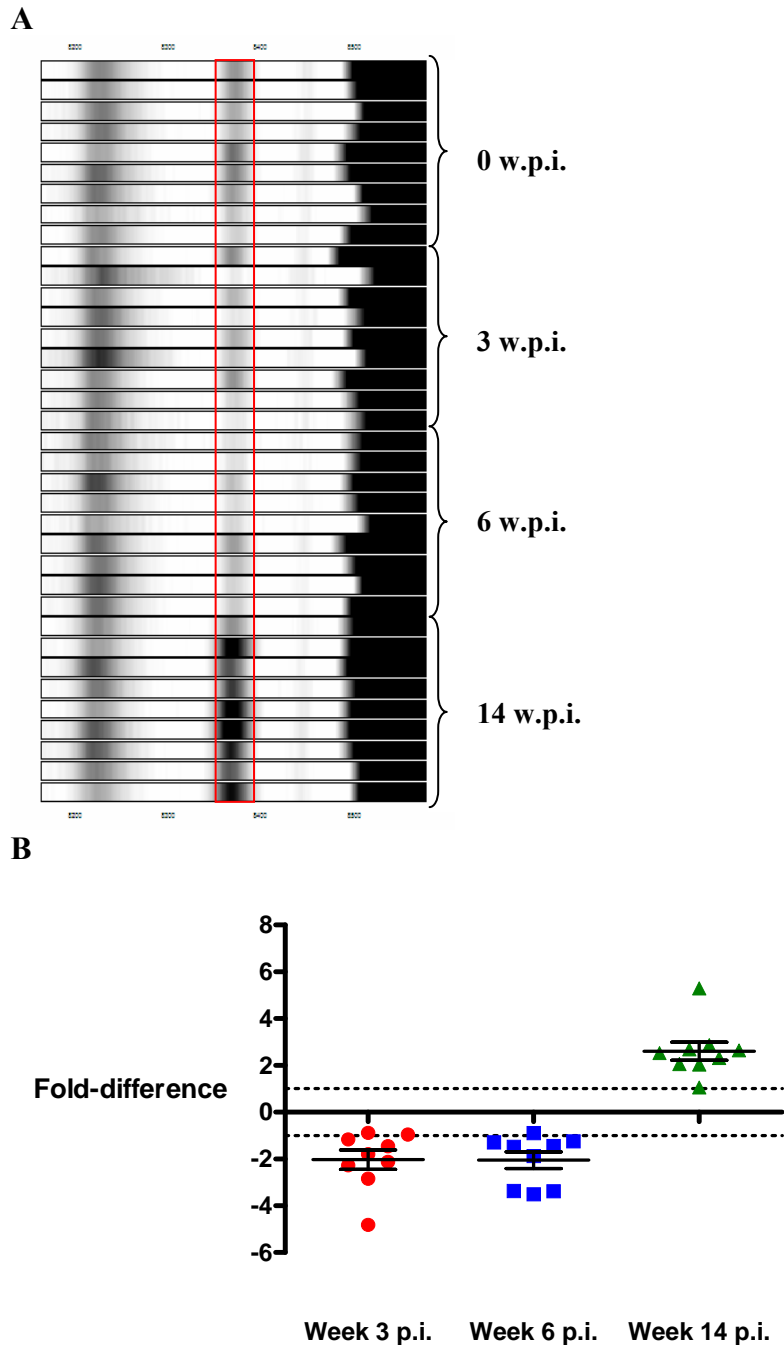


Figure 25: Statistical validation of 8364 Da marker of *F. hepatica* trickle infection identified in SELDI-TOF MS study

A trace at 8364 Da in the organic wash fraction was found to be significantly decreased at 3 and 6 w.p.i. and increased at 14 w.p.i. in two independent analyses using individual serum samples, as seen in the gel-view image of a set of representative SELDI-TOF MS (A) and fold-difference from paired control values (B).

3.3 Depletion study

The IgY-12 column removed transferrin, albumin, IgG heavy chain and IgG light chain as seen in the SDS-PAGE separation of low abundance serum proteins and high abundance serum proteins in three separate depletions compared to unprocessed serum (Fig. 26A). SELDI-ToF MS, being more accurate in the determination of the M/z of proteins, also clearly shows the depletion from serum of major high abundance proteins (Fig. 26B), and the concentration of the high abundance proteins in the eluted fraction (Fig. 26C).

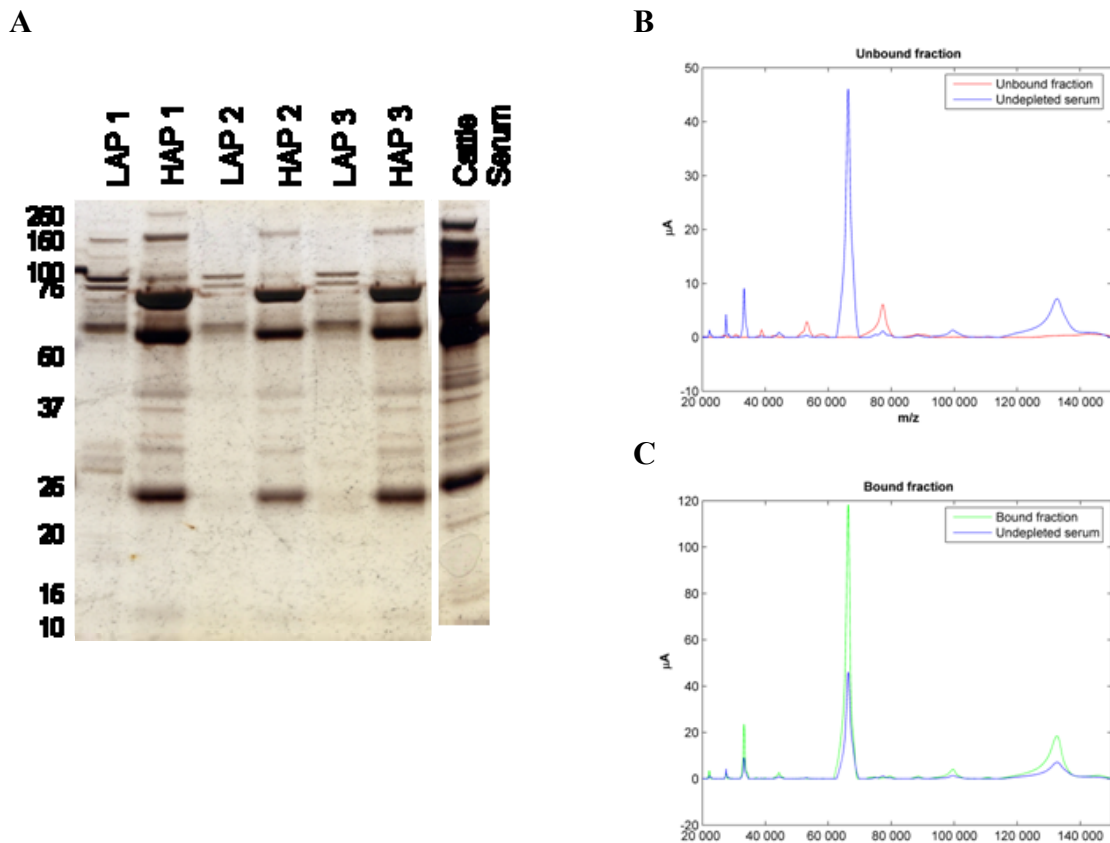


Figure 26: Effectiveness of IgY-12 column high abundance plasma protein depletion on cattle sera

Samples of low-abundance protein eluate (LAP) and high-abundance proteins (HAP) stripped from an IgY-12 column in three independent trials were separated by SDS-PAGE (A) and compared to undepleted cattle serum (cattle serum). Samples were also spotted onto a gold chip and analyzed by PCS 4000 SELDI-TOF MS (B and C). The profiles of the unbound (LAP) fraction (B) and the bound fraction (HAP) (C) were compared to the profile of undepleted cattle serum.

3.4 MS/MS of depleted sera and markers of infection

Proteins in LAP samples of control sera and sera from 1-7 w.p.i. and 13-14 w.p.i. were fractionated by 1DE, individual gel slices collected, trypsinised and peptides analysed by MS/MS (Fig. 27). Fifty-five proteins were identified in the samples with two or more unique peptide in at least one sample. Similar numbers of proteins were identified in each group, with 45 proteins in the control sample, 44 proteins in the pool of sera from early infection (1-7 w.p.i.) and 44 proteins in the sera from chronic infection (13 and 14 w.p.i.).

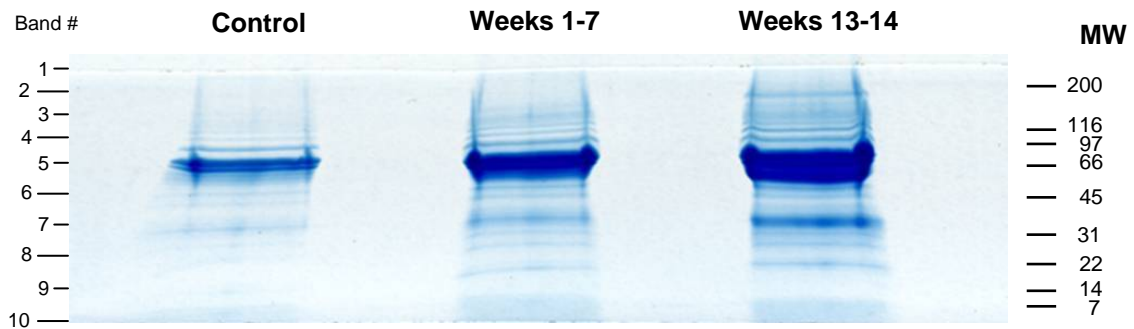


Figure 27: 1DE fractionation of depleted cattle serum samples

Samples from pools of cattle sera before infection (0 w.p.i.), during the early migratory phase (1-7 w.p.i.) and biliary stage (13-14 w.p.i.) of *F. hepatica* infection were separated by SDS-PAGE using a 7-15% acrylamide gradient.

Using the number of peptides as a measure of the relative intensity of the sample, serpin A3-1 and A3-5 demonstrated 3-fold increases, whereas haptoglobin was detected solely in the liver phase of infection (1-7 w.p.i.). α 2-macroglobulin and albumin had increased peptide counts in the chronic stage of infection (13-14 w.p.i.) (Table 10). Some proteins had increased levels in both stages of infection, including plasminogen, haemoglobin subunit beta, complement factor H, conglutinin and α 1-antitrypsin-like protein. IgG1 heavy chain had increased levels throughout infection and associated light chains

lambda and kappa chains were increased in the chronic stage of infection (Table 10).

Proteins with decreased peptide counts during infection were low-abundance proteins with peptide counts of 5 or less. Proteins with decreased levels during the acute stage of infection include apolipoprotein A-I, retinol-binding protein 4, α 1-microglobulin, which were not detected at weeks 1-7 p.i., and serum albumin, which showed a 5-fold decrease (Table 11). During the chronic phase, L-lactate dehydrogenase B chain and gelsolin showed 3-fold decreases; serpin clade A and vitamin D-binding protein were not detected during the chronic stage of infection (Table 11). Endopin 2B was not detected in the infection samples, it was found only in the control sample (Table 11).

Table 10: Positive markers of *F. hepatica* trickle infection in cattle

Proteins identified by LC-MS/MS in pooled serum depleted of high abundance proteins prior to infection (control), 1-7 w.p.i. (early liver stage) and 13-14 w.p.i. (acute bile duct stage). Proteins listed were found to have a 3-fold increase in the number of peptides as compared to the control group at 1-7 w.p.i. (A), 1-7 and 13-14 w.p.i. (B) or only in the pool of 13-14 w.p.i. (C).

*^a denotes an increase from undetectable amounts in the control sample

^b Serum albumin is a negative marker of infection at 1-7 w.p.i. and a positive marker of infection 13-14 w.p.i.

n.i., no increase, indicates that the difference between control and infected peptide counts was not above the established threshold

A

Description	UniProt	Species	Control	1-7 w.p.i.	Fold-increase	13-14 w.p.i.	Fold-increase
Haptoglobin	Q2TBU0	<i>Bos taurus</i>	0	8	* ^a	0	n.i.
Serpin A3-5	A2I7N1	<i>Bos taurus</i>	24	74	3.1	51	n.i.
Serpin A3-1	Q9TTE1	<i>Bos taurus</i>	32	97	3.0	68	n.i.

B

Description	UniProt	Species	Control	1-7 w.p.i.	Fold-increase	13-14 w.p.i.	Fold-increase
Immunoglobulin heavy constant gamma 1	A6H7A1	<i>Bos taurus</i>	0	3	* ^a	56	*
Complement factor H	Q28085	<i>Bos taurus</i>	0	7	* ^a	4	* ^a
α 1-antitrypsin-like protein	Q1KSC9	<i>Marmota monax</i>	0	1	* ^a	3	* ^a
Conglutinin	P23805	<i>Bos taurus</i>	0	2	* ^a	1	* ^a
Tetranectin	Q2KIS7	<i>Bos taurus</i>	0	1	* ^a	2	* ^a
Plasminogen	P06868	<i>Bos taurus</i>	2	8	4	23	11.5
Hemoglobin subunit beta	P04245	<i>Tragelaphus strepsiceros</i>	1	3	3	6	6

C

Description	UniProt	Species	Control	1-7 wpi	Fold- increase	13-14 wpi	Fold- increase
α 1-antitrypsin-like protein	Q1KSC9	<i>Marmota monax</i>	0	1	* ^a	3	* ^a
α 2-macroglobulin	A8E647	<i>Bos taurus</i>	8	10	n.i.	66	8.25
Immunoglobulin light chain, lambda gene cluster	Q2KIF5	<i>Bos taurus</i>	5	7	n.i.	36	7.20
	Q1RMN8		10	9	n.i.	54	5.40
Immunoglobulin kappa chain	Q05B55	<i>Bos taurus</i>	3	6	n.i.	15	5
Serum albumin	P02769	<i>Bos taurus</i>	5	1	n.i. ^b	28	5.6

Table 11: Negative markers of *F. hepatica* trickle infection in cattle

Proteins identified by LC-MS/MS in pooled serum depleted of high abundance proteins prior to infection (control), 1-7 w.p.i. (early liver stage) and 13-14 w.p.i. (acute bile duct stage). Proteins listed were found to have a 3-fold decrease in the number of peptides as compared to the control group at 1-7 w.p.i. (A), 1-7 and 13-14 w.p.i. (B) or only in the pool of 13-14 w.p.i. (C).

*^a denotes a decrease to undetectable amounts in the control sample

^b Serum albumin is a negative marker of infection at 1-7 w.p.i. and a positive marker of infection 13-14 w.p.i.

n.d., no decrease, indicates that the difference between the control and infected peptide counts was not above the established threshold

A

Description	UniProt	Species	Control	1-7 w.p.i.	Fold-decrease	13-14 w.p.i.	Fold-decrease
Apolipoprotein A-I	P15497	<i>Bos taurus</i>	3	0	*	7	n.d.
Retinol-binding protein 4	P18902	<i>Bos taurus</i>	3	0	*	4	n.d.
α 1-microglobulin	P00978	<i>Bos taurus</i>	2	0	*	2	n.d.
Serum albumin	P02769	<i>Bos taurus</i>	5	1	5	28	n.d. ^b

B

Description	UniProt	Species	Control	1-7 w.p.i.	Fold-decrease	13-14 w.p.i.	Fold-decrease
Endopin 2B	Q9TS74	<i>Bos taurus</i>	3	0	*	0	*

C

Description	UniProt	Species	Control	1-7 w.p.i.	Fold-decrease	13-14 w.p.i.	Fold-decrease
Vitamin D-binding protein	Q3MHN5	<i>Bos taurus</i>	4	2	n.d.	0	*
Serpin clade A	Q9TT36	<i>Bos taurus</i>	2	1	n.d.	0	*
L-lactate dehydrogenase B chain	Q5E9B1	<i>Bos taurus</i>	3	2	n.d.	1	3
Gelsolin	Q3SX14	<i>Bos taurus</i>	3	2	n.d.	1	3

4. Discussion

Identification of early markers of *F. hepatica* infection in cattle could inform our understanding of the host response to the initial stage of fasciolosis infection and provide potential markers for diagnosis using a SELDI-TOF MS platform. The proteomic profile during infection is complex, with 50 potential markers identified in the preliminary analysis of fractionated sera, and two biomarkers identified as statistically significant. Data obtained by SELDI-TOF MS were exported and the results compared using algorithms in MATLAB software to generate a robust list of candidate biomarkers that were most likely to be biologically relevant. Many markers were found to be significantly different using statistical analysis in Ciphergen 3.0.6 software, but only two of these markers corresponded to the M/z of the markers identified in the preliminary study. The main drawback of SELDI-TOF MS in proteomic profiling is the difficulty in identifying a protein responsible for the differences in the MS traces in complex samples. In this study, the purification and identification was not successful due to the low abundance of the markers in the samples.

In order to optimize chances to identify serum markers of *F. hepatica* infection, an alternative approach was used. Proteins were separated by 1DE and LC-MS/MS to compare the abundance of identified proteins during the course of infection. To increase the sensitivity of the technique (Echan et al., 2005; Greenough et al., 2004), IgY-12 columns (Beckman-Coulter) were used to deplete 12 high abundance proteins. This column contains chicken affinity-purified polyclonal antibodies raised against human proteins. The significant advantage of using IgY antibodies over IgG results from the evolutionary distance between chicken and mammals, allowing for greater immunogenicity against conserved mammalian proteins such as albumin (Hinerfeld et al., 2004). Columns produced with polyclonal antibodies can tolerate modest changes in sequence homology enabling a good depletion efficiency across a number of species (Cellar et al., 2008). Depletion was reproducible using a modified protocol (Fig. 26A), and increased the intensity of traces in SELDI-TOF MS (Fig. 26B, C), especially in

the albumin region. Bellei et al. (2010) also reported an improvement in SELDI-TOF MS traces in the albumin-region after IgY-12 depletion of human serum. The proteins detected using this approach included proteins in the $\mu\text{g/ml}$ range of serum proteins, including adiponectin, serum amyloid P and complement factor I, which have serum concentrations of 8.4, 25 and 25.5-51 $\mu\text{g/ml}$ in human serum (Hortin et al., 2008).

Circulating *F. hepatica* ES antigens have been detected in humans at a minimum concentration of 10 ng/ml by ELISA, with an average concentration of 32.0 ng/ml (Espino et al., 1990), and at a minimum sensitivity in sheep serum of 190 ng/ml (Almazan et al., 2001). Cathepsin L are the most abundant proteins in the *F. hepatica* ES released *in vivo* (Morphew et al., 2007) and are estimated to be released *in vitro* at a rate of 0.5-1.0 $\mu\text{g/ml/hour}$ (Dalton et al., 2006). In our study, we would expect the plasma cathepsin L concentration to be approximately 6.2-12.4 ng/ml, based on the amount of cathepsin L released by an average of 110 parasites in one hour in an estimated plasma volume of 8.9 L for a two-year old heifer (Dalton et al., 1996; Howes et al., 1963; Kalm et al., 1978). The proteins detected are in the moderate abundance range (8-51 $\mu\text{g/ml}$). The detection limits of this method were not sufficient to detect low abundance proteins such as parasite proteins, or host interleukins (in the pg/ml range).

Due to the tissue damage caused during the parasite migration during the acute liver stage of the infection, markers of the acute phase would be expected: haptoglobin, α 1-acid glycoprotein, serum amyloid A and fibrinogen are positive markers of the acute phase response, whereas albumin and transferrin are negative acute-phase proteins (Petersen et al. 2004; Murata et al. 2004; Crisman et al. 2008). Though not all acute-phase proteins are present in the analysis, increased levels of haptoglobin and decreased albumin were observed (Tables 2 and 3).

The maturation of *F. hepatica* parasites in cattle is associated with hepatic fibrosis and calcification of bile ducts. Positive markers of liver fibrosis have been reported to include α 2-macroglobulin (Gangadharan et al., 2007; White et al., 2007), haptoglobin (White et al., 2007), and immunoglobulin components (Gangadharan et al., 2007). Apo A-I and Apo A-IV have been reported to be

negative markers of infection (White et al., 2007). α 2-macroglobulin, albumin and Apo A-I have been used in a fibrosis diagnostic test (White et al., 2007). In this study, α 2-macroglobulin and immunoglobulin components increased during the chronic stage of infection whereas Apo A-I decreased during infection.

Components of the innate and adaptive immune system were important elements of the proteomic profile in both the acute and chronic stages of infection. Notably, complement factor H, conglutinin and immunoglobulin components were increased in both stages of infection. Complement factor H was increased in both infected samples. Complement factor H regulates the alternative activation of complement pathway by inactivating C3b, thus inhibiting convertase formation, and delays the acceleration of the convertase once formed on host cells. The protein is synthesized in the liver and its expression is induced in inflammation (Friese et al., 2000; Zipfel et al., 2007), and may help minimize tissue damage. Conglutinin is a collectin expressed in cattle and is involved in the alternative activation of complement (Gjerstorff et al., 2004), acts as an opsonin and an acute-phase reactant in chickens (Murata et al., 2004). Immunoglobulin components were increased in both acute and chronic stages of infection. Notably, IgG1 was increased throughout infection. IgG1, an antibody associated with a type 2 response, is present in the immune response to a natural *F. hepatica* infection (Mulcahy et al., 1998). This finding acts as a proof-of-concept in demonstrating that MS/MS spectrum-counting can identify markers of infection.

Many of the markers of infection are protease inhibitors. α 1-antitrypsin is a broad class of serine protease inhibitors (serpins) that includes the A3-1, A3-5 and A3-7 (endopin 2B) serpin classes. Serpin A3-1 and A3-5 control elastase activity and have been reported to increase in inflammation (Gettins, 2002). These serpins were increased in the acute phase of infection (Table 10), a phase marked by significant tissue damage and an early inflammatory response. *F. hepatica* infections are associated with a switch to a type 2 response approximately 4 w.p.i.; a successful establishment of infection by the parasite requires down-regulation of the inflammatory response. Levels of A3-1 and A3-5 return to normal levels during the chronic stage of infection, corresponding to this down-

regulation of the immune response (Table 10). α 2-macroglobulin was increased in chronic infection (Table 10). α 2-macroglobulin inhibits serine, cysteine, aspartic and metalloproteases and is responsible for controlling coagulation and fibrinolysis. It may function to inhibit parasite-derived proteases. Endopin 2, classified as an A3-7 class serpin, and vitamin D-binding protein, classified as class A7 serpin, both had decreased peptide counts during infection. Class A3-7 serpins completely inhibit papain and elastase (Hwang et al., 2002). Decreased endopin 2 levels were found in both the acute and chronic stages of infection. Additional proteins of interest include plasminogen, vitamin D-binding protein and gelsolin. Plasminogen was increased throughout infection. Plasminogen leads to the formation of plasmin, which dissolves the fibrin of blood clots and acts as a proteolytic factor in a variety of processes, including inflammation and complement activation. The increase in plasminogen may be associated with the immune response to infection and repair of damaged tissue. Vitamin D-binding protein and gelsolin, both found to be decreased in the chronic stage of infection, are also both involved in the clearance of actin (Dahl et al., 1999; Schiodt, 2008). Decreased plasma gelsolin concentrations have been reported in trauma patients (Dahl et al., 1999; Lee et al., 2007). Vitamin D-binding protein is cleaved by elastase *in vivo* (Jirasakuldech et al., 2000) and has been reported to be decreased in the serum of septic patients (Afandi et al., 2000). Both gelsolin and vitamin D-binding protein may therefore be associated with tissue damage.

This study presents two approaches to the proteomic profiling of serum proteins from cattle trickle-infected with *F. hepatica*. SELDI-TOF MS profiling shows a range of peaks that are associated with infection, but immunodepletion of high abundance proteins combined with 1DE and LC-MS/MS analysis of pooled samples allowed for a more descriptive proteomic profile of the host-parasite interaction. The technique is not currently sufficiently sensitive to detect parasite proteins, but this non-biased approach to the profiling of biomarkers successfully identified markers of the acute phase response, liver fibrosis and immunomodulation by the parasite. Differences in the relative abundance of host protease inhibitors were prominent in our findings, and their effects on the

cathepsin L, B and leucine aminopeptidase enzymes in the ESP of the parasite warrant further study.

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Chapter 6: Summary and conclusions

Fasciolosis has an important economic impact in the veterinary sector. *F. hepatica* is considered more virulent than *F. gigantica* (Raadsma et al., 2007) because of its ability to establish primary infections in definitive hosts. Certain hosts have the ability to resist a second *F. hepatica* infection. By comparing responses to *F. hepatica* infection in susceptible animals, such as sheep, and resistant animals, such as cattle, indicators of an effective response can potentially be identified and used to monitor responses in vaccine trials. In addition, comparing NEJ and adult ESP proteins profiles from *F. hepatica* and *F. gigantica* parasites will provide a basis for understanding the difference in virulence between the two species and identify common vaccine or chemotherapeutic targets.

Serum protein indicators of the host response to *F. hepatica* infection in sheep were profiled using a non-biased proteomic approach. Sera from sheep infected with single doses of *F. hepatica* metacercariae were profiled using SELDI-TOF MS, providing an overview of the changes in spectra for the first twelve weeks of infection. Twenty-six spectral traces were identified as markers of infection. Two of these biomarkers were successfully purified and identified: transferrin, a 77.0 kDa marker, and apolipoprotein A-IV, a 44.3 kDa marker. Both transferrin and apolipoprotein A-IV markers were upregulated during the chronic stage of infection at 9 w.p.i. The increase of both markers was confirmed by Western blot analysis, providing a proof-of-concept that biomarkers identified by SELDI-TOF MS can be validated using alternate techniques.

In order to compare the host responses to *F. hepatica* infection, we performed similar SELDI-TOF MS study in trickle-infected cattle. The direct comparison of these two studies was limited by the nature of the samples available for analysis: the study of biomarkers in sheep consisted of sera from animals experimentally infected with a single dose of metacercariae, allowing a uniform maturation time course of parasites in the host, whereas the study of biomarkers in cattle simulated a natural infection with a trickle-dose infection, where the development of the parasites was staggered in time with the infectious

doses of metacercariae. The two studies therefore provide different insights into the pathogenesis of the virulent parasite in susceptible and resistant hosts, respectively.

The response to *F. hepatica* trickle-infection in cattle was investigated using a combination of SELDI-TOF MS and LC-MS/MS analysis of sera from infected animals. The analysis of SELDI-TOF MS spectra was refined after the first study of infected sheep sera (Chapter 3) by introducing a mathematical algorithm to identify peaks with differences in relative abundance within defined ranges in the preliminary study. This eliminated the subjective nature of the analysis of spectra from pooled samples. The statistically significant peaks identified using MS spectra analysis software were compared to the list of potential markers identified in the preliminary study for validation. These methods resulted in the identification of two markers of infection: a marker at 1852 Da that was significantly decreased at 6 and 10 w.p.i., corresponding to the acute/mature stage of infection, and a marker at 8364 Da that was significantly decreased at 3 and 6 w.p.i., corresponding to the acute stage of infection, and significantly increased at 14 wp.i., corresponding to the chronic stage of infection. Due to the size and low abundance of the markers of infection, the proteins corresponding to these spectral traces could not be successfully purified and identified. In order to address the limitations of the SELDI-TOF MS technology and to be able to give a comprehensive overview of the changes in serum proteome profile in cattle during *F. hepatica* infection, serum proteins were separated using SDS-PAGE and the proteins were identified by LC-MS/MS. Immunoaffinity depletion of the sera increased the relative abundance of low abundance proteins in cattle sera, demonstrating the effectiveness of the human IgY-12 columns on heterologous proteins in cattle sera. The combination of immunoaffinity depletion and MS/MS analysis permitted the identification of moderate abundance markers, including indicators of inflammation and liver fibrosis, though it was not sufficiently sensitive to identify parasite proteins. Indicators of inflammation, complement factor H, serpin A3-1, serpin A3-5 and plasminogen were found during the acute phase of the infection, with increased

levels of complement factor H and plasminogen during the chronic stage of infection. Indicators of liver fibrosis, including increased α 2-macroglobulin and immunoglobulin and decreased apolipoprotein AI, were found in the chronic stage of infection. These indicators of inflammation and liver fibrosis were identified using a non-biased approach. These indicators could be used in addition to GLDH and GGT as indicators of inflammation and fibrosis, respectively. The specificity of the technique was also able to identify specific protein isotypes, including increased IgG1 levels, an antibody isotype that has been associated with a non-protective immune response to *F. hepatica* in cattle (Mulcahy et al., 1998) that results from the immunomodulation of the parasites. Conglutinin, associated with the alternative activation of macrophages, was also found to increase during infection (Gjerstorff et al., 2004). Conglutinin is part of a group of collectins found only in Bovidae, thought to be involved in innate immune responses to pathogens. The involvement of conglutinin in the response to fasciolosis *in vitro* and *in vivo* warrants further study. The role of host protease inhibitors in fasciolosis pathogenesis also warrants further study, specifically with respect to the effect on the enzymatic activity of parasite proteases and parasite development, and differences in expression of protease inhibitors between susceptible and resistant host species.

With the increased availability of *Fasciola spp.* genome sequences and availability of sensitive protein MS techniques, attention has focused on identifying proteins in ESP of *Fasciola spp.* and determining the clade specificity of the proteins released by the parasites at the NEJ, juvenile and adult stages (Jefferies et al., 2001; Morphew et al., 2011; Morphew et al., 2007; Robinson et al., 2009). Our comparative study of ESP protein profiles from NEJ and adult stages of *F. hepatica* and *F. gigantica* is the first comprehensive analysis of ESP from *F. gigantica*. The differences in ESP profiles are greater between the life-stages of the parasites than between the two species. The most notable difference in expression profiles between the two life stages were the types of proteases released by the parasites, and the isotype specificity of the proteases released. Antioxidant defence enzyme levels were more abundant in adult ESP than NEJ

ESP, but higher relative levels of these enzymes were found in *F. gigantica* than *F. hepatica*.

ESP proteases in NEJ and adult samples differed, with isotype differences noted as well. Proteases are the most abundant class of proteins released by NEJ and include cathepsin L, cathepsin B and legumain in relatively equal abundance. The principal protease released by adult parasites was cathepsin L. Cathepsin L isotypes detected in the samples were clearly stage-specific, with clades A, F, and G detected in NEJ, and clades B, C, D and E in adults. Clade F was specific to *F. gigantica* NEJ. Given that the S2 active site sequences for NEJ and adult isotypes differ, the substrate specificity of these enzymes should be investigated using recombinant proteins, as recently described (Norbury et al., 2010). These findings are also important for vaccine development, as candidate vaccines should incorporate potential epitopes that are NEJ-specific and cross-reactive between NEJ and adult isotypes. Potential vaccines should include epitopes from many isotypes in order to increase protection rates. If used as a chemotherapeutic target, the diversity of active site configurations should be taken into consideration during testing to ensure efficacy against diverse cathepsin L proteases released by the parasites. Similarly, given that aspariginyl endopeptidase (Legumain SmAE) has been found to increase FhCatB1 activation *in vitro* (Beckham et al., 2006), the high proportionate expression of clade 3 cathepsin B with an NEJ-specific legumain sequence (CAC85636) in NEJ may be used in combination as vaccine candidates targeted to interfere early in fasciolosis pathogenesis.

Antioxidant defence enzymes were identified in all ESP samples, including thioredoxin-related proteins involved in the detoxification of reactive oxygen species (Selkirk et al., 1998) and immunomodulation (Donnelly et al., 2005). Thioredoxin peroxidase was highly abundant in all samples analysed, and presents a promising chemotherapeutic target or vaccine antigen. Antioxidant defence enzymes are more abundant in adult ESP samples, with higher relative abundance in *F. gigantica* than *F. hepatica*. Glutathione *S*-transferases were found only in adult samples. Previous characterizations of *Fasciola* spp. GST were limited to mu-class enzymes, with the recent isolation of sigma-class GST

enzymes in *F. hepatica* (Chemale et al., 2006). Our studies suggest that the principal GST enzymes in ESP in both *Fasciola* species are sigma-class GST, followed by mu-class GST. We also found evidence of expression of a putative omega-class GST in adult *F. gigantica* ESP. In addition to GST, Cu/Zn-SOD was found in all ESP samples analysed, with the higher relative abundance in NEJ, with higher relative abundance in *F. hepatica* NEJ than *F. gigantica* NEJ, as previously reported (Piedrafita et al., 2007). The role of antioxidant defence proteins in the development of both parasite species warrants further attention.

FABP, a current vaccine candidate (Hillyer, 2005), also showed both stage- and species-specific isotype profiles. NEJ FABP was limited to type 3 isotype. Adult FABP in *F. hepatica* and *F. gigantica* differed markedly with FABP types 1, 2, and 3 detected in *F. hepatica* ESP, but only type 1 FABP was detected in *F. gigantica* ESP. The functional differences between these isotypes warrants further study, and attention to isotype specificity should be considered in further vaccine studies.

In conclusion, these proteomic analyses of the host-parasite interactions of fasciolosis have expanded our understanding of the host response to infection, and have also given insight into the similarities and differences between the two parasite species. In addition, these studies present progress that has been made in validating the tools and techniques used in the analysis of animal sera and parasite ESP. The results highlight the differences in protein expression between the life stages of the parasites and the diversity of protein isotypes expressed in each sample, which are important in the development of vaccines targeted to early stages of infection and development of broad-spectrum chemotherapeutic agents.

Chapter 6 References

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