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Exploring and Expanding the Fatty Acid Binding Protein superfamily in *Fasciola* species

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Running title. FABP superfamily in *Fasciola* Species

Abbreviations: TCBZ, triclabendazole; FABP, fatty acid binding protein;

Keywords: *Fasciola hepatica*; *F. gigantica*; Proteomics; Diagnosis; Gene Characterisation

Abstract

The liver flukes *Fasciola hepatica* and *F. gigantica* infect livestock worldwide and threaten food security with climate change and problematic control measures spreading disease. Fascioliasis is also a food borne disease with up to 17 million humans infected. In the absence of vaccines, treatment depends on Triclabendazole (TCBZ) and over-use has led to widespread resistance, compromising future TCBZ control. Reductionist biology from many laboratories has predicted new therapeutic targets. To this end, the fatty acid binding protein (FABP) superfamily have proposed multi-functional roles, including functions intersecting vaccine and drug therapy, such as immune modulation and anthelmintic sequestration. Research is hindered by a lack of understanding of the full FABP superfamily complement. Although discovery studies predicted FABPs as promising vaccine candidates, it is unclear if uncharacterised FABPs are more relevant for vaccine formulations. We have coupled genome, transcriptome and EST data mining with proteomics and phylogenetics, to reveal a liver fluke FABP superfamily of 7 clades: previously identified clades I-III and newly identified clades IV-VII. All new clade FABPs were analysed using bioinformatics and cloned from both liver flukes. The extended FABP dataset will provide new study tools to research the role of FABPs in parasite biology and as therapy targets.

Introduction

The trematode liver flukes, *Fasciola hepatica and F. gigantica*, are the causative agents of fasciolosis, a foodborne zoonotic disease affecting grazing animals and humans worldwide. Liver fluke causes economic losses of over US$ 3 billion worldwide per annum to livestock via mortality, reduction in host fecundity, susceptibility to other infections, decrease in meat, milk and wool production and condemnation of livers (1). Liver fluke disease of livestock is increasing worldwide (2), with a number of potential contributing factors: climate change (warmer winters and wetter summers supporting larger intermediate mud snail host populations); fragmented disease management (only treating sheep not cattle and limiting veterinary interaction); encouragement of wet-lands; livestock movement; and/or failure/resistance of chemical control treatments in the absence of commercial vaccines (3, 4). Fasciolosis is also a re-emerging human disease with estimates of between 2.4-17 million people infected worldwide (5-7). Furthermore, worldwide livestock movement is providing new opportunities for the introduction of pathogenic isolates (4).

Control of liver fluke is currently via anthelmintics. The benzimidazole (BZM)-derivative, triclabendazole (TCBZ), is the drug most extensively used against *Fasciola.* Unlike other fasciolicides, TCBZ shows activity against both juvenile flukes, which are responsible for the damage to the liver of acute fasciolosis, and the mature flukes which cause the debilitation of chronic fasciolosis (8). However, TCBZ resistant liver fluke are increasing throughout Europe and Australia, compromising control efforts (9-12). Following end of patent protection, generic forms of TCBZ will likely lead to wider application, potential misuse and exacerbation of the spread of resistance.

The mode of action of TCBZ at the molecular level has yet to be resolved. Laboratories report a variety of biological effects of TCBZ on liver fluke (for reviews see 9, 13). As well as increased efflux (14), enhanced biotransformation and metabolism of TCBZ has also been hypothesised to play a major role in detoxification and resistance. Anthelmintic resistance can arise from efficient detoxification via Phase II and Phase III conjugation, sequestration and efflux mechanisms. To this end, a type I FABP, Fh15 protein with sequestration potential is significantly upregulated on TCBZ exposure in resistant adult liver fluke (15) and a FABP from *Schistosoma japonicum* has been reported to play a role in Praziquantel drug binding (16). Moreover, increased expression of FABP mRNA occurs in drug resistant strains of *Anopheles gambiae* during permethrin insecticide exposure (17). Thus, increased expression of FABP maybe a generic invertebrate response to drug challenge and a potential resistance marker.

The soluble super family of fatty acid binding proteins (FABPs) are small (14–15 kDa) proteins that bind or sequestrate hydrophobic ligands such as anthelmintics (16). The precise function of each FABP type remains imperfectly understood, since sub-specialization of functions is suggested. At least nine distinct types of cytoplasmic FABPs have been identified in mammals, each showing a characteristic pattern of tissue distribution (18). FABPs isolated from the same tissue of different vertebrate species show sequence identities of 70 % and higher, whereas FABPs isolated from different tissues of a single species have sequence similarity as low as 20 %. However, their tertiary structure is remarkably conserved, consisting of ten anti-parallel β-strands comprising a β-barrel (containing conserved amino acid residues that are involved in ligand binding) and a helix-turn-helix cap (16).

Importantly for vaccine candidature and drug sequestration, FABP is also an abundant component of the soluble tegumental proteome of adult liver fluke (19, 20). Vaccination against liver fluke remains at the research stage (21, 22). Previous studies have placed cytosolic FABPs amongst the major potential vaccine candidates (23). Vaccine trials suggest that both native and recombinant *F. hepatica* FABPs induce significant levels of protection in different animal models against infection with *F. hepatica* and cross-protection against *S. mansoni* and *S. bovis* with anti-fluke, anti-fecundity and anti-pathology effects (23).

The recent discovery of FABPs in the cargo of exosome-like vesicles released from adult *F. hepatica* (24), suggests new roles for parasite FABPs within host cells. In support of this hypothesis *in vitro* assays demonstrate FABP I (Fh12) alters behaviour of monocyte derived macrophages, with increased arginase expression/activity and an increase in chitinase-3-like protein (25). FABP I also down regulated nitric oxide production and the expression of nitric oxide synthase in interacting cells exhibiting a potent anti-inflammatory effect in inducing the production of alternatively activated macrophages (25). Furthermore, FABP I has been shown to suppress inflammatory cytokines in a model of septic shock potentially delivering its effect via binding to CD14 co-receptors (26).

Despite growing evidence supporting the importance of FABPs for the establishment of liver fluke in the vertebrate host, our knowledge of the superfamily complement is fragmented even with many reductionist studies. We report the mining of the genome, transcriptomic and EST datasets, supplemented with proteomics and phylogenetics, to systematically reveal the complexity and novelty within the liver fluke FABP superfamily. Furthermore, we have identified which of these FABPs are recognised by the immune system for further vaccine discovery.

Experimental Procedures

Fluke collection and cultures

Adult *F. hepatica* were recovered from naturally infected ovine livers immediately post-slaughter from a local abattoir in Mid-Wales, UK. Fluke were washed several times in PBS at 37 °C to remove host material by regurgitation of gut contents as previously described (27). Adult flukes were transported to the laboratory and maintained in *Fasciola* saline (FS; Dulbecco's Modified Eagle's Medium (DMEM) [w/o $NaHPO_3$ and PO_4] plus 2.2 mM Ca [$C_2H_3O_2$], 2.7 mM $MgSO_4$, 61.1 mM glucose, 1 µM serotonin, 5 $\mu g ml^{-1}$ gentamycin, 15 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4) at 37 °C for 2 hrs. Metacercariae were purchased from Ridgeway Research and excysted following the physiological method outlined by Dixon (28, 29) and described previously (30).

Cytosol preparations and 2DE

F. hepatica extracts were obtained by homogenisation of frozen fluke at 4°C in lysis buffer containing 20 mM Potassium phosphate, pH 7.4, 0.1% (v/v) Triton-X100 and protease inhibitors (Roche, Complete-Mini, EDTA-free). After homogenisation, samples were centrifuged at 100,000 × *g* for 1 h at 4°C and the supernatant was termed the cytosolic fraction. Cytosolic protein extracts were precipitated with an equal volume of ice-cold 20% TCA in acetone (w/v) and washed twice in ice-cold acetone before solubilisation into isoelectric focusing buffer (IEF buffer) consisting of 8 M urea, 2% w/v CHAPS, 33 mM DTT and 0.5% carrier ampholytes v/v (Biolyte 3-10, Bio-Rad) as described previously (31). For preliminary 2DE, a total of 100 µg was passively in-gel rehydrated for 16 h and isoelectrically focused on 7 cm linear pH 3-10 IPG strips (BioRad) for 10,000 Vh. For analytical 2DE a total of 500 µg of each replicate sample was passively in-gel rehydrated for 16 h and isoelectrically

focused on 17 cm pH 4.7-5.9 or 7-10 IPG strips (BioRad) to 60000 Vh. All IEF was conducted on a Protean® IEF Cell (Bio-Rad).

After focusing, strips were equilibrated for 15 min in reducing equilibration buffer (30% v/v glycerol, 6 M urea, 1% DTT) followed by 15 min in alkylating equilibration buffer (30% v/v glycerol, 6 M urea, 4% iodoacetamide). IPG strips were run upon SDS-PAGE (14 % acrylamide) using the Protean® II xi 2D Cell (BioRad) for 17 cm or PROTEAN® Mini (Bio-Rad, UK) for 7 cm 2DE. Gels were Coomassie blue stained (Phastgel Blue R, Amersham Biosciences) and scanned on a GS-800 calibrated densitometer (BioRad).

Gel image quantitative differences between protein spots were analysed via Progenesis PG220 software, version 2006 (Nonlinear Dynamics). Spots were manually detected on gels with normalisation performed using total spot volume multiplied by 100. Quantitative analysis was based on average gels created from four biological replicates.

Protein identification

Protein spots were manually excised and tryptically digested and prepared for mass spectrometry as previously described (32, 33). Tandem mass spectrometry (MS/MS) was performed according to the method of Moxon *et al.* (34) followed by data processing for database searching. Samples prepared for liquid chromatography–tandem mass spectrometry (LC–MS/MS) were analysed using electrospray ionisation as previously reported (34). Peptide mixtures from trypsin digested gel spots were separated using a LCPackings Ultimate nano-HPLC System. Sample injection was via an LC Packings Famos auto-sampler and the loading sol-vent was 0.1% formic acid. The pre-column used was a LCPackings C18 PepMap 100, 5 mm, 100 A and the nano HPLC column was a LC Packings PepMap

C18, 3 mm, 100 A. The solvent system was: solvent A (2% ACN with 0.1% formic acid), and solvent B (80% ACN with 0.1% formic acid). The LC flow rate was 0.2 µL/min with a gradient employed using 5% sol-vent A to 100% solvent B in 1 h. The HPLC eluent was sprayed into the nano-ES source of a Waters Q-TOFµMS via a New Objective Pico-Tip emitter. The MS was operated in positive ion mode and multiply charged ions were detected using a data-directed MS/MS experiment. Collision induced dissociation (CID) MS/MS mass spectra were recorded over the mass range m/z 80–1400 Da with scan time 1 s. MassLynx v 3.5 (Waters, UK) ProteinLynx suite of tools was used to process raw fragmentation spectra. Each spectrum was combined and smoothed twice using the Savitzky–Golay method at ±3 channels with background noise subtracted at polynomial order 15 and 10% below curve. Monoisotopic peaks were centred at 80% centroid setting. Sequest compatible (.dta) file peak mass lists for each spectrum were exported, and spectra common to each 2DE spot were merged into a single MASCOT generic format (.mgf) file using the on-line Peak List Conversion Utility available at www.proteomecommons.org. Merged files were submitted to a MASCOT MS/MS ions search within a locally installed Mascot server (www.matrixscience.com) to search an 'in-house' database constructed from 6260 (858 763 residues) *F. hepatica* EST sequences downloaded and translated from the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/ pathogens/fasciola/hepatica/ESTs/). Search parameters were as described in Morphew *et al*. (33).

Western blotting

For Western blotting samples were prepared and separated according to the method described above. Proteins were transferred to HybondTM-C Extra nitrocellulose membrane (GE Healthcare) using a Trans-blot Cell (Bio-Rad) at 20 V

overnight. Transfer was carried out in 192 mM glycine (Sigma), 25 mM Tris base and 20 % (v/v) methanol according to the method of Towbin *et al*. (35). The membrane was stained for 1 min 10 with 0.1 % (w/v) amido black 10B (Naphthol Blue Rack, Sigma) in 10 % (v/v) acetic acid and 25 % (v/v) isopropanol and destained in 10 % (v/v) acetic acid and 25 % (v/v) isopropanol to evaluate transfer efficiency.

NC membranes were blocked in TTBS (0.1 M Tris base, pH = 7.5, 0.9 % (w/v) NaCl, 1 % (v/v) TweenR 20 (Acros)) containing 5 % (w/v) skimmed milk powder for a minimum of 4 h. Membranes were then washed in TTBS for 10 min before being incubated for 1 h with the primary antibody diluted at 1:5,000 in TTBS containing 1 % (w/v) skimmed milk powder. In all Western blots pooled, bovine, whole sera from *Fasciola hepatica*-challenged and naive cattle were used as described previously (36).

Membranes were washed three times for 5 min in TBS (0.1 M Tris base, pH = 7.5, 0.9 % (w/v) NaCl, 1 % (v/v)) to remove residual primary antibody and incubated for 1 h with anti-bovine IgG (whole molecule) conjugated to alkaline phosphatase (AKP, Sigma) diluted at 1:30,000 or anti-bovine IgM (whole molecule) conjugated to AKP (Novus Biologicals) diluted at 1:1,000 both in TTBS containing 1 % (w/v) skimmed milk powder. The membranes were again washed three times for 5 min in TBS. Visualisation of the resulting immuno-complexes was achieved by developing the membranes in 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma) and nitro blue tetrazolium (NBT; BDH, VWR Life Sciences) according to the manufacturer's instruction.

Bioinformatics -Transcripts and Phylogenetics

All sequence alignments were carried out using ClustalW multiple alignment (37) via BioEdit Version 7.0.9.0 (6/27/07) (38). Previously published FABP

sequences from both *Fasciola* species were retrieved from the Genbank database (http://www.ncbi.nlm.nih.gov/) and aligned with ESTs and transcripts from available sources (39)(ftp://ftp.sanger.ac.uk/pub/project/pathogens/Fasciola/hepatica/ESTs/; EBI-ENA archive ERP000012: An initial characterization of the *F. hepatica* transcriptome using 454-FLX sequencing). Transcripts and ESTs that matched at least one of the already known FABPs in a BLAST analysis were included in alignments. From the alignment a phylogenetic tree was constructed in MEGA v 4.0 using a neighbor-joining method, 1000-replicate, bootstrapped tree. The amino acid data were corrected for a gamma distribution (level set at 1.0) and with a Poisson correction.

Bioinformatics - Analysis of novel FABPs

Secondary structure analysis of novel FABP isoforms was conducted using PSIPRED available at http://bioinf.cs.ucl.ac.uk/psipred/. Motif analysis was conducted using InterPro (40) and phosphorylation predictions using NetPhos 2.0 (41). Sequences from *F. hepatica* and *F. gigantica* encoding novel FABP isoforms were also subjected to epitope predictions using a Kolaskar and Tongaonkar Antigenicity prediction method (42), available at http://tools.immuneepitope.org/tools/bcell/iedb_input, and signal peptide analysis using the Signal P 4.1 server and a cut-off point of 0.45 (43). Intron-exon structures of novel *F. hepatica* FABP isoforms was also determined using Artemis (44) on sequences identified in the *F. hepatica* genome (45).

Cloning

Novel FABP isoforms from *F. hepatica* and *F. gigantica* were cloned using primers designed on *F. hepatica* and *F. gigantica* transcripts (FABP IV: forward primer 5′ ATG GAA GCA TTC GTC GGA 3′ and reverse primer 5′ TCA AAT TTT CTG GAA

TTT GAA G 3′. FABP V: forward primer 5′ CGG GTC TCT GCC CTG TAT ATT 3′ and reverse primer 5′ TGT GAC GGG ATA AAC CCA AT 3′. FABP VI: forward primer 5′ TCG CCA TAT TGG TAC ATT 3′ and reverse primer 5′ CAT TTA ATG GGC GCC GCT 3′. FABP VII: forward primer 5′ TCA ACC ATG TCA AAG CTT AT 3′ and reverse primer 5′ GAC AAG CGG GTA CAT TCA TG 3′ or 5′ GAC AAG CTT GTA CAT TCA TG 3′). *Fasciola* FABP sequences were amplified using PCR. Both *F. hepatica* and *F. gigantica* sequences were then cloned into the pGEM-T easy vector (Promega) according to manufacturer's instructions, screened and sequenced in-house.

Recombinant protein production and purification

FABP IV and V from both fasciolids were amplified from plasmids containing inserts with the addition of *Nde*-I and *Not*-I restriction enzyme sites for directional cloning into the pET28a (Novagen) expression vector. Recombinant FABP protein was expressed via the *Escherichia coli* BL21(DE3) and pET28a expression vector system (Novagen) and purified using nickel-affinity via a C-terminal polyhistadine tag as previously described (31). Purity was assessed by electrospray ionisation (ESI) mass spectrometry and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Characterisation of FABP from *F. hepatica* ontogeny

In order to effectively resolve the known FABP isoforms present in *F. hepatica*, both adult and newly excysted juveniles (NEJ) were subjected to preliminary 2D SDS-PAGE analysis using broad range IPG strips to localise FABP representatives. Based upon previous 2D SDS-PAGE investigations into the FABP superfamily of *Fasciola* (15, 19, 36), the comparison of NEJ and adult 2D arrays revealed a dramatic reduction of FABP isoforms in NEJs (Figure 1a). This absence of FABPs from the NEJ proteome included the slightly acidic FABPs, isoforms I and II (Figure 1a i), and the basic isoform, FABP III (Figure 1a ii). As a result of these preliminary proteomes further FABP isoform delineation was to be conducted upon adult samples using selective to IPG strips (pH 4.7-5.9 for acidic FABPs and pH 7-10 for basic FABPs) to provide maximum resolution.

FABP sub-proteome of adult *F. hepatica*

Separation of adult *F. hepatica* somatic homogenates on narrow and micro range IPG strips and subsequent SDS-PAGE resulted in identification of FABP isoforms belonging to the type I, II and III sub-families. A total of 15 protein spots were consistently identified via Progenesis analysis lying below the 20 kDa marker and occupying the recognised FABP 'zones' (Figure 1b). MSMS data from the peptides excised from 4.7-5.9 micro range gels (Figure 1c) confirmed that FABP II isoforms appear in four different locations on the gel, varying in p*I* as well as in molecular weight (Table 1). FABP I (Fh15) isoform was found in three locations of the same gel analysis. A discrepancy in p*I* and molecular weight between values calculated by Progenesis (5.3- 5.6/17.4-20.5 kDa) and those predicted from Genbank entries (5.93/14936.04 Da for FABPII, Q7M4G1 and 5.91/14712.08 Da for

Fh15, Q7M4GO) was observed. To account for multiple FABP locations, potential sites of post-translational phosphorylation were investigated and identified five Serine and Threonine residues for FABP I (Fh15) and three for FABP II predicted with high significance to be phosphorylated.

MSMS data from the peptides excised from pH 7-10 narrow range gels (Figure 1d) confirmed that FABP III is the most abundant FABP isoform and was identified in five different locations on the gel, varying principally in p*I* (Table 1). FABP II was identified in two further locations and in both cases co-localised with FABP III. Of interest was the solitary identification of FABP III co-localised with a serine/threonine protein kinase. As with FABP I and II, FABP III potential sites of post-translational phosphorylation were investigated identifying three serine and one threonine residues predicted with high significance to be phosphorylated. Only two protein spots identified did not contain FABP isoforms.

Potential Immune recognition of FABP isoforms

Having identified the isoforms of FABP present in adult *F. hepatica*, somatic homogenate samples were then subjected to 1D Western blotting using a pooled bovine infection sera time course to reveal the dynamics of the FABP superfamily members and the host IgG response. Somatic homogenate samples were probed with time course sera from week 0, naïve animals, to week 14 post infection (excluding week 10 post infection) to look for FABP interaction. Data showed that an IgG response to proteins of the appropriate sizes could be observed from as early as 2 weeks post infection (Figure S1A) with two bands visualised. The extent of this IgG recognition persisted until 14 weeks post infection. In order to determine if any of the FABP isoforms (FABP I, II or III) were responsible for the IgG recognition all

samples were subjected to 2DE profiling as described previously followed by Western blotting.

The 2DE array, spanning pH 4.7-5.9, of the cytosolic fraction of adult liver fluke homogenate was transferred to NC membranes and assayed with sera from infected and naive cattle. Pooled sera from week 8 post infection produced the strongest IgG response and thus selected to probe 2DE Western blot arrays. The transfer of somatic samples to membranes for antibody testing was assessed using amido black staining (Figure S1b). Once probed with week 8 sera only very weak IgG responses were seen. This recognition was primarily to spots containing FABP I (Figure S1c, spots 2, 4 and 5 in Figure 1c) and extremely weakly to those spots containing FABP II (Figure S1c, spots 6, 7 and 8 in Figure 1c). Importantly, the strongest, albeit weak, IgG response was to spot 2 which contained both FABP I and a translation initiation inhibitor (Figure S1c, spot 2 in Figure 1c). Thus, it is likely that the immune recognition of FABPs I-III is extremely low. Importantly, naïve sera (week 0) showed no IgG response (Figure S1d). IgG responses were also highlighted in an unresolved section of the gel at the p*I* 5.9 side of the gel.

The 2-DE array (pH 7-10) of the cytosolic fraction of adult liver fluke homogenate was transferred to NC membranes and assayed with sera from infected and naive cattle. As with acid FABP isoforms week 8 infection sera was chosen to screen the basic putative FABPs for immunoreactivity. However, no antibody recognition could be detected for any of the basic FABP isoforms or indeed any proteins lower than the 30 kDa marker (Data not shown).

Transcript and phylogenetic analysis of FABP isoforms

Available transcript and EST data sets from both *F. hepatica* and *F. gigantica* and the *F. hepatica* genome were searched for representative FABP superfamily

members. All identified members representing known FABP isoforms I, II and III and potential new FABP isoforms were aligned with members of the known vertebrate FABP classes. From the multiple alignment a phylogenetic tree was constructed to ascertain the *Fasciola* FABP clade structure. The previously identified *Fasciola* FABP I, II and III clades were well conserved and most related to clades from the known vertebrate Heart (H, B, A, T and M-FABP) and Intestinal (I and K-FABP) FABP groups (Figure 2). The FABP type I clade comprises the recombinant FABP I (Fh15) from *F. hepatica* and reveals a clear separation between representatives of both *Fasciola* species into two subclades. This subclade structure is not replicated in clades II or III as there is a lack of *F. gigantica* FABP II representatives and the sequences of FABP III from both species are 100% identical at the amino acid level. A fourth novel clade (Bootstrap support 90%), termed FABP V, clustered near the known three isoforms but much closer to the known vertebrate classes, in particular the vertebrate keratinocyte and intestinal FABP isoforms. Interestingly, the FABP Sm14 from *S. mansoni* clustered close to *Fasciola* FABP clade V. Three further novel clades of *Fasciola* FABPs were identified with strong bootstrap support, termed FABP IV (99%), VI (91%) and VII (96%; Figure 2). All three of these putative novel FABP clades, as with FABP clade I, also showed a distinct separation between representatives of *F. hepatica* and *F. gigantica*. Both FABP clades VI and VII were most related to the vertebrate liver and ileal forms of vertebrate FABPs with FABP clade IV to a lesser extent.

Representatives of the novel four FABP clades from both *F. hepatica* and *F. gigantica* were cloned and sequenced. This revealed proteins of 132 (FABP IV), 134 (FABP V), 162 (FABP VI) and 166 (FABP VII) amino acids in length. The average amino acid sequence similarity to the previously identified *Fasciola* FABPs ranged

from as low as 22% to 48%. Specifically, FABP IV was most similar to FABP I (24-25%), FABP V to FABP III (48%), FABP VI to FABP II (24-25%) and FABP VII to FABP III (22%). Initially, all sequencing was performed on adult cDNA however expression of all 4 novel FABPs was confirmed in NEJ cDNA (Data not shown). All *F. hepatica* and *F. gigantica* clones for the novel FABP classes are included in Figure 2.

Confirmation of novel FABP isoforms

All four newly identified putative FABP sequences were confirmed as FABPs using bioinformatics. As an initial comparison, the gene structures of the four new isoforms were compared to those of FABPs I, II and III (Figure 3a). This analysis revealed that only the structure of FABP IV matched those of I-III. FABP V only differed by 6 bp (3 bp in exon 2 and 3 bp in exon 4). Of note were the dramatic increase in size of exon 4 in both FABP VI and VII.

All novel FABP isoforms were then subjected to secondary structure prediction analysis to identify the characteristic ten anti-parallel β-strands comprising a β-barrel and the helix-turn-helix cap. All novel FABP isoforms from both *F. hepatica* and *F. gigantica* were predicted to have the FABP characteristic structure (10 β-strands and 2 α–helices; Figure 3b). All four novel isoforms were also subjected to InterPro sequence analysis for FABP domain predictions. In all cases, both IPR011038 Calycin-like and IPR012674 Calycin were predicted for both *Fasciola* species isoforms. Furthermore, three of the four isoforms (excluding FABP IV) were predicted to have IPR000463 Cytosolic fatty-acid binding properties. Of note was a well conserved cytosolic fatty-acid binding signature in all novel FABP isoforms; in particular FABP V with 94.4% sequence identity to the recognised motif signature (Table 2; GKWKLVDSR***D***FDKVMVEL). All four novel sequences were also

subjected to a SVMProt prediction to identify functional characterisation. Novel FABPs were all identified as Lipid binding proteins, further confirming their status as FABPs, with the exception of FABP VI. In this instance FABP VI was identified as a zinc binding protein. As expected, all novel FABP isoforms lacked a signal peptide further confirming them as cytosolic FABP isoforms. Importantly, both N-terminal residues G6 and W8 are conserved in all novel FABP isoforms.

Expression of FABP isoforms IV and V

Due to their similarity to the three previously known FABP isoforms both FABP IV and V from *F. hepatica* and *F. gigantica* were chosen for expression in *E. coli* for immunological characterisation. Both proteins from both species were purified using Ni^{2+} affinity chromatography and assessed for purity using 1D and 2D SDS-PAGE in conjunction with ESI-MS for an accurate mass assessment. All proteins were purified to a high level as assessed by both methods (Figure 4). Both FABP IV (predicted molecular weight 15,073 Da and 16,351 Da with the Histag) and V (predicted molecular weight 15,372 Da and 16,650 Da with the Histag) had commonly observed Na^+ adduct formation (mass shifts of 22 Da between peaks) and a shift of 63 Da from the expected molecular weight relating to ammonium formate ($NCOONH_4$) adduct formation during ESI-MS analysis (Figure 4A). FABP V was observed as a more basic isoform from 2D SDS-PAGE (approximate p*I* 8.4; Figure 4C) than that of FBAP IV (Figure 4B). Minor shifts from the predicted molecular weights were seen for both isoforms which may indicate lipid binding during purification as no delipidation steps were incorporated.

Both expressed FABP isoforms were assessed for serum antibody responses using a time course of *Fasciola hepatica*-challenged and naïve bovine sera (weeks 0-14 post infection). FABP IV showed no IgM or IgG responses throughout a 14 week

infection (Data not shown). However, FABP V from both *F. hepatica* and *F. gigantica* had observable IgM and IgG responses as assessed by Western blotting (Figure 5). IgM responses towards FABP V were visualised strongly at week 4-6, whereas IgG responses were visualised earlier at weeks 2-4.

A total of 41 *Fasciola* individuals were subjected to an analysis of FABP V to identify any potential sequence variation within the population (Data not shown). This included 33 adult *F. hepatica* from 3 different populations (South Gloucester, Camarthen and Llanidloes; all UK) and 8 adult *F. gigantica* (North and South India). Upon analysis, only a few single amino acid polymorphisms (SAAPs) were identified between all *Fasciola* spp. FABP V sequences. This was limited to just four of the individuals sequenced with a total of 4 SAAP sites. A single SAAP site was localised in two individual *F. hepatica* from the Llanidloes population (Q66L and Y130N), a further SAAP was identified in a Northern India *F. gigantica* sample (Q66R) and finally two SAAPs in one *F. gigantica* sequence from the South of India (V69I and K87R). Interestingly, 3 of the 5 SAAPs identified (those at sites 66 and 69) fall within a predicted epitope that spans amino acid residues 63-70.

Absence of Novel FABP Isoforms in Exosome-like Vesicles As both FABP II and III have been previously identified in *F. hepatica* exosome-like vesicles (24) proteomic data was searched for all novel FABP isoforms. Interestingly, none of the novel FABP isoforms were identified in exosome-like vesicles (Pers. comm. Marcilla June 2014).

Discussion

Reductionist studies have proposed diverse roles in parasitism for FABP isoforms, including uptake/transport of fatty acids as there is limited synthesis in helminth worms, immune modulation via their fatty acid ligands and the sequestration of anthelmintics as a resistance mechanism. However, in order to effectively investigate the function of the FABPs it is imperative to first resolve and fully delineate the complexity and novelty within the superfamily. Hence, we have utilised a polyomics approach incorporating genomics, transcriptomics and proteomics to reveal a FABP superfamily from the pathogenic liver fluke parasite *F. hepatica* with seven distinct isoforms; four more than previously discovered.

Our previous observations revealed a dominance of FABP abundance in adult liver fluke rather than the more pathogenic NEJs, where FABPs were significantly reduced in abundance. Relatively low levels of FABPs in juvenile liver fluke compared to adults is replicated in other parasitic platyhelminths and thus not simply related to adult survival in the liver/bile environment. For example, in schistosomes an increase of FABPs is observed from newly developed schistosomula through to lung stage schistosomula and with the greatest abundance of FABPs found in adults (46, 47) suggesting FABPs are important for development. The differential expression of FABPs from *F. hepatica* may be explained by functional roles; namely intracellular transport and detoxification. Intracellular FABPs function as fatty acid (FA) transport proteins (16, 18). Therefore, in the absence of FA synthesis in adult parasitic flatworms, high levels of FABPs may transport FAs that are taken up via uncharacterised tegumental mechanisms from the host environment (48). However, within NEJs there is a high level of preformed/stored lipid to support initial host survival (49, 50) and likely a reduced requirement for fatty acid uptake and FABP

transporters, hence their dramatic reduction observed in 2DE arrays (Figure 1A). The greater abundance of adult liver fluke FABPs may be related to adult feeding patterns. At the onset of blood feeding it has been suggested that FABPs are essential for the uptake of FAs from host blood (51). Furthermore, FABPs sequestrate and remove haem, a toxic by-product of blood feeding responsible for hydrogen peroxide (H_2O_2) production if in the free form (52). In support of this theory, antibodies directed towards *F. hepatica* FABPs can be observed around 2-4 weeks post infection (53 and the present study) coinciding with migration through the liver and the onset of blood feeding behaviour.

Delineating the adult FABP proteome revealed the three known *Fasciola* FABP isoforms, reported in the recent publication of the *F. hepatica* genome (45), in multiple locations within the 2DE arrays (3 locations for FABP I, 6 for FABP II and 5 for FABP III); an observation often causing confusion over true isoform identification (54). So why are the *F. hepatica* FABP isoforms resolving into numerous protein spots and will this impact upon future diagnostics or vaccines? Multiple resolved versions of FABP isoforms have been attributed to post translational modification, especially phosphorylation (55), and tyrosine phosphorylation is known to inhibit/modulate the binding of FAs (56). In mammals, phosphorylation of FABPs appears low >1% (54) but bioinformatics predicts multiple phosphorylation sites in *Fasciola* FABPs, tentatively suggesting a difference in FA transport regulation between host and parasite. In contrast, multiple resolved versions of FABP isoforms could also be attributable to irreversible ligand or FA binding (54).

Resolving the function of each FABP isoform is compounded by the complex diversity, tissue and temporal specificity and ligand preferences of each FABP isoform (16). For example, FgFABP I and FgFABP III from *F. gigantica* have some

overlapping roles yet FgFABP I supports the male reproductive system and FgFABP III supports the female reproductive system (57). It is likely that this distribution of FABPs I and III will be replicated within *F. hepatica* tissues. In the current study, *F. hepatica* FABP isoforms I-III were assessed for host immune recognition. Mirroring the study of Chunchob *et al.* (57) no immune recognition was observed to FABP isoform III using bovine sera. However, recognition of a protein spot containing FABP isoform I was seen, unlike for FgFABP I. However, the strongest, albeit weak, immune recognition identified was to a protein spot containing both FhFABP I and translation initiation inhibitor (TII). Given the lack of immune recognition to other FhFABP I protein spots in the array the primary response seen may relate to TII. This is especially pertinent with the identification of TII in *F. hepatica* tegument preparations, both S2SS and UTCS fractions, of the study by Wilson *et al.* (20) and in surface preparations, both SPF and IPF protein fractions, from the study of Harcariz *et al*. (58). It is likely that TII is exposed to the host immune system and thus elicit the immune response identified in the current study.

We performed a bioinformatic analysis of currently available transcript and genomic databases for *F. hepatica* and *F. gigantica* to probe for the first time the complexity of the liver fluke FABP superfamily. As with previous phylogenetic studies FABP isoforms I, II and III formed a distinct group close to the vertebrate H- and I-FABP groups. FABP I isoforms had a clear separation related to *Fasciola* species not seen in FABP II or FABP III isoform groupings due to limited sequence data availability and 100% amino acid sequence identity between fasciolids respectively.

Further phylogenetic analysis identified four new FABP isoforms represented in both *F. hepatica* and *F. gigantica* and in both adults and NEJs, namely isoforms IV-VII. All four of these novel isoforms were confirmed as FABPs using bioinformatics. Only

FABP isoform VI did not conform to every bioinformatics analysis predicting a FABP; namely a SVMProt prediction did not identify FABP VI as a Lipid binding protein predicting a Zinc binding protein instead. In this case, FABP VI was classified as a FABP on its gene structure (exons 1-3 matching known FABP exon structures), protein structure (10 β-strands and 2 α-helices) and its highly conserved cytosolic FA binding domain signature (88.9% amino acid identity). Phylogenetically, isoforms IV, VI and VII were split between *F. hepatica* and *F. gigantica* as seen with isoform I. Using bioinformatic analysis all novel sequences were confirmed as authentic FABP isoforms by analysing gene structures, sequence motifs and secondary structure prediction.

FABP isoform V was closely related to isoforms I-III but closer to vertebrate I- and K-FABPs. Vertebrate I-FABPs are generally known to bind FAs only rather than additional ligands. In addition, FAs bound to I-FABPs are also bound in a different conformation to the other vertebrate FABPs (FAs bind to I-FABPs in a bent conformation instead of a U shaped conformation when bound to alternative vertebrate FABPs) (59). Therefore, it is possible that FABP V is expressed specifically for the uptake of FAs from the host, although binding similarities to I-FABPs will require confirmation as the ligand binding residues of I-FABP (Y70, L72, A73, W82, Q115 and Y117) are not conserved in FABP isoform V (59).

The three other novel *Fasciola* FABP isoforms, IV, VI and VII, clustered away from the previously known *Fasciola* FABPs and were located close to the vertebrate L- and IL-FABP groups. As with the FABP I, II and III isoforms we would expect to identify multiple versions upon a 2DE array. The potential of cysteine modification via glutathionylation or cysteinylation, related to the redox state of the host liver, may complicate distinguishing true FABP isoforms as seen for vertebrate L-FABP (54).

Vertebrate L-FABPs are distinctly different from other vertebrate FABP groups. Firstly, they differ in the FA uptake mechanism, which in L-FABPs occurs via diffusion (16) rather than collision as demonstrated for H- and I-FABPs. Furthermore, as a result of their large binding pocket, ligands of L-FABPs bind at a 2:1 molar stoichiometry ratio as opposed to 1:1 in all others examined (60). Importantly, vertebrate L- and IL- FABPs are capable of binding FAs as well as bulky ligands such as bile salts, cholesterol and haem (61). Whilst all of these abilities remain to be confirmed in the novel *Fasciola* FABP isoforms IV, VI and VII, it would seem logical that these isoforms could well be adapted for a 'life in bile' as a blood feeder. Interestingly, two of the novel FABP isoforms, VI and VII, had a 31 and 33 amino acid C-terminal extension respectively. Whilst the role of these extensions is currently unknown they may well be involved in interactions or attachments to other proteins or for membrane association (62).

It has been suggested that a reduced diversity of FABP isoforms in invertebrates compared to vertebrates represents a lower specificity for ligands but a larger repertoire of interactions within the cell (16). In contrast, the number of FABP isoforms in Fasciolids has expanded suggesting potential specialisation of FABP isoforms. The vertebrate groupings H-, I- and L-FABPs reflect their specific binding abilities (61). Thus, it is likely that *Fasciola* FABPs may also segregate according to their binding abilities. The separation of FABP isoforms I, II, III and V from isoforms IV, VI and VII may represent two clusters based upon their respective binding capabilities.

Disappointingly, none of the newly recognised FABP isoforms (IV-VII) were identified during proteomic analysis. This could potentially result from the quantity of protein in the identified protein spots, identified as FABPs I, II and III, supressing any

recognition of the novel FABP isoforms. Alternatively, it may be that FABPs IV-VII may be of more importance in the juvenile stages of the parasite such is the case with cathepsin proteases (cathepsin L1, 2 and 5 in adults whereas cathepsin L3 and 4 with cathepsin B in juveniles) (27).

FABP isoforms IV and V were expressed as recombinant forms in order to further understand the immune responses directed to the FABP family for diagnostic or vaccine potential. Despite its absence from *F. hepatica* exosome-like vesicles, FABP isoform V shows potential as a diagnostic or vaccine candidate with strong IgM and IgG responses seen in pooled bovine infection sera. As diagnostics, *Fasciola* FABPs show promise with two studies by Allam and colleagues suggesting FABP for the diagnosis of *F. gigantica* infections in both buffalo and man (63, 64). In both cases FABPs were purified from crude adult worm extracts, likely to contain multiple FABP isoforms. Thus, FABP isoforms I-III are likely to dominate the preparation but the presence of FABP IV-VII cannot be discounted. Furthermore, Hillyer *et al*. (53) also noted that antibodies to FABP I could also be observed at 2-4 weeks post infection highlighting the excellent potential of FABPs as diagnostics. However, to further improve diagnostic potential the correct choice of which FABP isoform to target is essential. For example, from the evidence presented in the current study, poor recognition to FABP I-III in natural infections and strong IgM and IgG responses to FABP V from weeks 2-4, FABP isoform V may be a potential choice as a diagnostic. However, with the expanded FABP family, from three to seven members, each isoform must be investigated specifically to gauge the best choice for a diagnostic.

As vaccine candidates, FABPs have also been studied in depth. Early studies using FhFABPs looked promising with trials in mice revealing 69-78% protection.

Unfortunately, these protection rates did not translate well into cattle trials with two studies giving 31 and 55% protection (Vaccine trials reviewed by 65). Poor translation into target animals was also observed in trials for protection against *F. gigantica.* For example, in buffalo a 35.8% reduction in worm burdens with associated reductions in liver enzymes (AST) was observed (66). However, this trial also demonstrated a high anti-fecundity effect (66). As with other candidate vaccines based upon protein superfamilies the choice of which isoform to vaccinate with is key decision ensuring plasticity of the target is low (27). Therefore, *Fasciola* FABP isoform V may be a strong candidate for novel vaccine trials perhaps as part of a combination therapy. A potential combination therapy could incorporate FABP V and an immune suppressor component, such as FhGST-S1 (19, 67). FABP potential could also be enhanced by conducting similarity studies between those isoforms eliciting an immune response (FABP isoforms I, II and V) which may reveal the structural details that are responsible for triggering this immune response in the host organism. This could lead to the rational design of protein complexes that may prove to be more effective in vaccine trials than current vaccine candidates.

Conclusions

A poly-omics approach has successfully revealed the cytosolic FABP superfamily complement expressed in *F. hepatica adults*. These have been classified into seven isoforms, types I-VII, with a potential clustering of isoforms into two groups; group one consisting of isoforms I, II, III and V and group two consisting of isoforms IV, VI and VII. These two groupings may reflect their binding dynamics. Importantly, FABP isoform V from both *F. hepatica* and *F. gigantica* shows promise as a new diagnostic antigen or as a vaccine candidate.

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Supporting Information

Table S1. Full coverage of the putative protein identifications of *F. hepatica* fatty acid binding proteins using MASCOT.

Figure S1. IgG antibody responses to known *F. hepatica* FABP isoforms using Western blotting

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Table 1. Putative protein identifications of *F. hepatica* fatty acid binding proteins using MASCOT. Spectra from mass spectrometry were subjected to MSMS ion searches using MASCOT (Matrix Science) searching an in house EST database. Significant hits, at P = 5%, have a MASCOT score of 25 or greater. All significant EST hits were then subjected to BLAST analysis against GenBank to assign an identity to matching ESTs. Therefore, all reported accession numbers are from GenBank. NS: Not significant.

Table 2. Fatty acid binding signature of novel fatty acid binding proteins. The fatty acid binding signature of all four novel FABPs from both *F. hepatica* and *F. gigantica* are highly conserved, especially so for FABP V. Residues shaded grey show 100 suitability to the signature requirements. A percentage match is given for the 18 residue motif.

Figure 1. Representative global protein arrays of *F. hepatica* FABPs. *F. hepatica* ontogeny 2D SDS-PAGE protein arrays of a) newly excysted juvenile and b) adult *F. hepatica*. Circled areas within the arrays localise known FABP isoforms I, II and III. Therefore, narrow range (pH 7-10) and micro range (pH 4.7-5.9) IPG strips were chosen as the most suitable range to effectively resolve the *F. hepatica* FABP members (denoted by dashed lines). Both arrays were loaded with 250 µg of cytosolic protein and run on linear pH 3-10 IPG strips and on 14% acrylamide SDS-PAGE. Both arrays were Coomassie blue stained. c) Representative micro range (pH 4.7-5.9) and d) narrow range (pH 7-10) protein arrays of adult *F. hepatica* somatic samples for FABP location and identification. Both c and d were loaded with 1 mg of cytosolic protein, run on 14% acrylamide SDS-PAGE and Coomassie blue

stained. Circled spots correspond to those proteins consistently present on averaged gels and their putative protein identifications can be found in Table 1.

Figure 2. Phylogenetic analysis of vertebrate and Fasciola FABPs to determine the clade structure of FABP isoforms present in *F. hepatica* and *F. gigantica*. Neighbor-joining phylogenetic tree constructed using amino acid sequences through MEGA v 4.0 with 1000 bootstrapped support and a Poisson correction. All reported accession numbers are from Genbank. Where sequences were identified *in silico*, only contig numbers are reported. Those from *F. gigantica* were taken from the study of Young *et al*. (39) and those from *F. hepatica* were taken from Young *et al*. (39) or transcripts produced by the University of Liverpool (EBI-ENA archive ERP000012: An initial characterization of the *F. hepatica* transcriptome using 454-FLX sequencing) Sequences from *F. gigantica* NEJs were sequenced in house.

Figure 3. Characterisation of novel FABP isoforms. A) Bioinformatic characterisation of *F. hepatica* fatty acid binding protein gene structures. All three currently identified FABP isoforms (I, II and III) were identified within the *F. hepatica* genome (45) and there intron-exon structures identified. These were compared to those of the four novel FABP isoforms (IV, V, VI and VII). Exons shaded in grey indicate deviation from those structures identified within isoforms I, II and III. Reported exon sizes are in nucleotide bp. B) Fatty acid binding protein secondary structure prediction. A multiple alignment of all four novel FABP isoforms from both *F. hepatica* and *F. gigantica* were subjected to secondary protein structure prediction to identify the FABP characteristic structure containing 10 β-strands and 2 α–helices. Predictions were carried out using PsiPred Version 3.2 (68). Each β-strand or α–helix is boxed

and numbered. The extended C-terminal of isoforms VI and VII are apparent with additional β-strand or α–helix predictions shown in grey shaded dashed boxes or open dashed boxes respectively. The three domains that constitute the lipocalin binding domain are boxed in blue. Arrowed are the starting and ending residues of the IPR000463 Cytosolic fatty-acid binding domain signature within domain 1. The GXW triplet domain in domain 1 is underlined in green. All predicted phosphorylation sites (S, T and Y) are boxed in red.

Figure 4. Expression of recombinant protein forms of novel FABP isoforms A) Purification of FABP IV from *F. hepatica* with purity revealed via ESI mass spectrum of the Ni^{2+} affinity purified rFhFABP IV showing the molecular weight at 16287.7 ± 0.49 Da (with 6 sodium adduct peaks). Data for FABP V not shown. B) Purity was also assessed with a 2D SDS-PAGE protein array run on 14% SDS-PAGE and Coomassie blue stained. C) Purification of FABP V from *F. hepatica* assessed with a 2D SDS-PAGE protein array run on 14% SDS-PAGE and Coomassie blue stained.

Figure 5. Antibody responses to novel *Fasciola* FABP isoform V. Representative Western blots of rFhFABP V samples looking for both IgM and IgG responses rFhFABP V was run on 14% acrylamide SDS-PAGE, electro-transferred to membranes and Western blotted with pooled bovine infection sera from week 0, naïve sera, to week 14 post infection as the primary antibody with anti-bovine IgM (a) or anti-bovine IgG (b) as the secondary antibody. A *F. hepatica* somatic sample (S) was also included and as a positive control using sera from week 8 post infection. White arrows indicate the start of immune recognition. Black arrow highlights the *F. hepatica* cathepsin L proteases recognised by bovine IgG (36).

Table 1

Spot	Mascot Score	EST Number	BLAST Score	Accession Number	Putative Identification	Species	Sequence Coverage	Unique Peptides	Nominal Mass	p/	Fasciola FABP Clade
1	260	Fhep06b08.q1k	1.00E-38	XP_002576820	Translation initiation inhibitor	*Schistosoma mansoni*	66	4	10043	5.13	-
	85	HAN5013c02.q1kT3	2.00E-45	XP_002576820	Translation initiation inhibitor	*Schistosoma mansoni*	26	2	12840	4.71	-
2	357	Fhep06b08.q1k	1.00E-38	XP_002576820	Translation initiation inhibitor	*Schistosoma mansoni*	66	4	10043	5.13	-
	149	HAN5013c02.q1kT3	2.00E-45	XP_002576820.1	Translation initiation inhibitor	*Schistosoma mansoni*	26	2	12840	4.71	-
	68	Fhep50d03.q1k	6.00E-56	Q7M4G0	Fatty acid-binding protein Fh15 (Type I)	*F. hepatica*	11	1	9367	6.26	I
3	88	Fhep06b08.q1k	1.00E-38	XP_002576820	Translation initiation inhibitor	*Schistosoma mansoni*	36	2	10043	5.13	-
	52	Fhep20f11.q1k	8.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	49	7	15327	5.21	II
4	265	Fhep46d02.q1k	1.00E-80	Q7M4G0	Fatty acid-binding protein Fh15 (Type I)	*F. hepatica*	39	5	19484	6.33	I
	194	Fhep50d03.q1k	6.00E-56	Q7M4G0	Fatty acid-binding protein Fh15 (Type I)	*F. hepatica*	54	4	9367	6.26	I
5	99	Fhep50d03.q1k	6.00E-56	Q7M4G0	Fatty acid-binding protein Fh15 (Type I)	*F. hepatica*	61	5	9367	6.26	I
	54	Fhep46d02.q1k	1.00E-80	Q7M4G0	Fatty acid-binding protein Fh15 (Type I)	*F. hepatica*	43	6	19484	6.33	I
6	227	Fhep20f11.q1k	8.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	65	9	15327	5.21	II
	208	HAN5004a01.p1kaT7	1.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	62	10	14869	5.63	II
7	109	Fhep20f11.q1k	8.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	65	8	15327	5.21	II
8	307	Fhep20f11.q1k	8.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	77	10	15327	5.21	II
	248	HAN5004a01.p1kaT7	1.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	68	10	14869	5.63	II
9	115	HAN3004-1f09.p1k	2.00E-89	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	38	4	14550	7.82	III
	77	Fhep20f11.q1k	8.00E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	29	3	15327	5.21	II
10	107	HAN3004-1f09.p1k	2E-89	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	38	4	14550	7.82	III
	29	Fhep20f11.q1k	8E-89	Q7M4G1	Fatty acid-binding protein Type II	*F. hepatica*	13	1	15327	5.21	II
11	112	HAN3004-1f09.p1k	2E-89	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	45	5	14550	7.82	III
12	153	HAN3004-1f09.p1k	2E-89	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	55	6	14550	7.82	III
	153	HAN5010e10.q1kT3	3.00E-87	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	61	7	14531	6.73	III
13	109	HAN3004-1f09.p1k	2E-89	Q9U1G6	Fatty acid-binding protein Type III	*F. hepatica*	31	3	14550	7.82	III
	30	Fhep08b02.q1k	5.5	ZP_06970083	Serine/threonine protein kinase	*Ktedonobacter racemifer*	9	1	8693	8.74	-
14	46	Fhep42c03.q1k	6.00E-15	Q5DC69	10 kDa heat shock protein	*Schistosoma japonicum*	56	2	4688	4.81	-
	43	HAN5022f11.q1kT3	2.00E-54	AAP06016	SJCHGC01960 protein (10 kDa heat shock protein)	*Schistosoma japonicum*	61	5	10799	9.02	-
15	NS	-	-	-	-	-	-	-	-	-	-

Table 2

	Signature requirement																	
	+	-	+		+			-		+	+	+		+	+	-	-	+	% Motif
Sequence	GSAIVK	FE	FYW	X	LIVMF	X	X	K	X	NHG	FY	DE	X	LIVMFY	LIVM	N	G	LIVMAKR	Match
Fhep FABPIV	G	K	W	K	L	D	S	Y	E	N	V	D	A	I	L	N	M	L	88.9
Fgig FABPIV	G	K	W	K	L	D	S	Y	E	N	V	D	A	I	L	N	M	L	88.9
Fhep FABP V	G	K	W	K	L	V	D	S	R	D	F	D	K	V	M	V	E	L	94.4
Fgig FABP V	G	K	W	K	L	V	D	S	R	D	F	D	K	V	M	V	E	L	94.4
Fhep FABPVI	G	E	W	E	A	T	G	H	R	N	F	S	S	I	L	A	E	I	83.3
Fgig FABPVI	G	E	W	E	A	T	G	Q	E	N	F	S	S	I	L	A	E	I	83.3
Fhep FABPVII	G	E	W	K	C	V	E	C	S	N	L	E	P	V	M	I	E	I	83.3
Fgig FABPVII	G	E	W	K	C	V	E	C	S	N	L	E	P	V	L	I	E	V	83.3

For Table of Contents Only

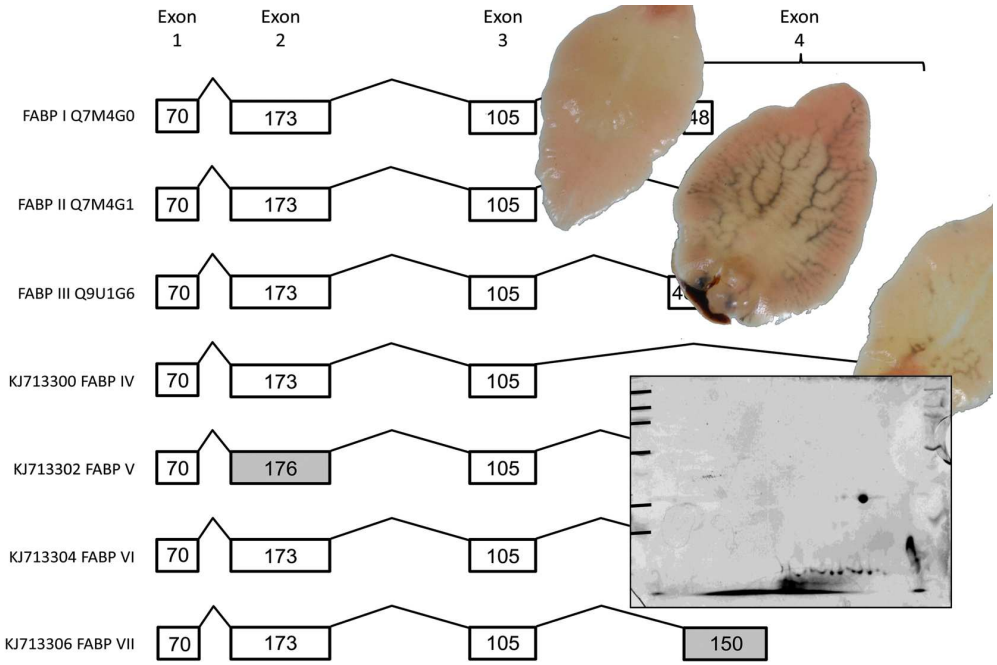

Figure 1

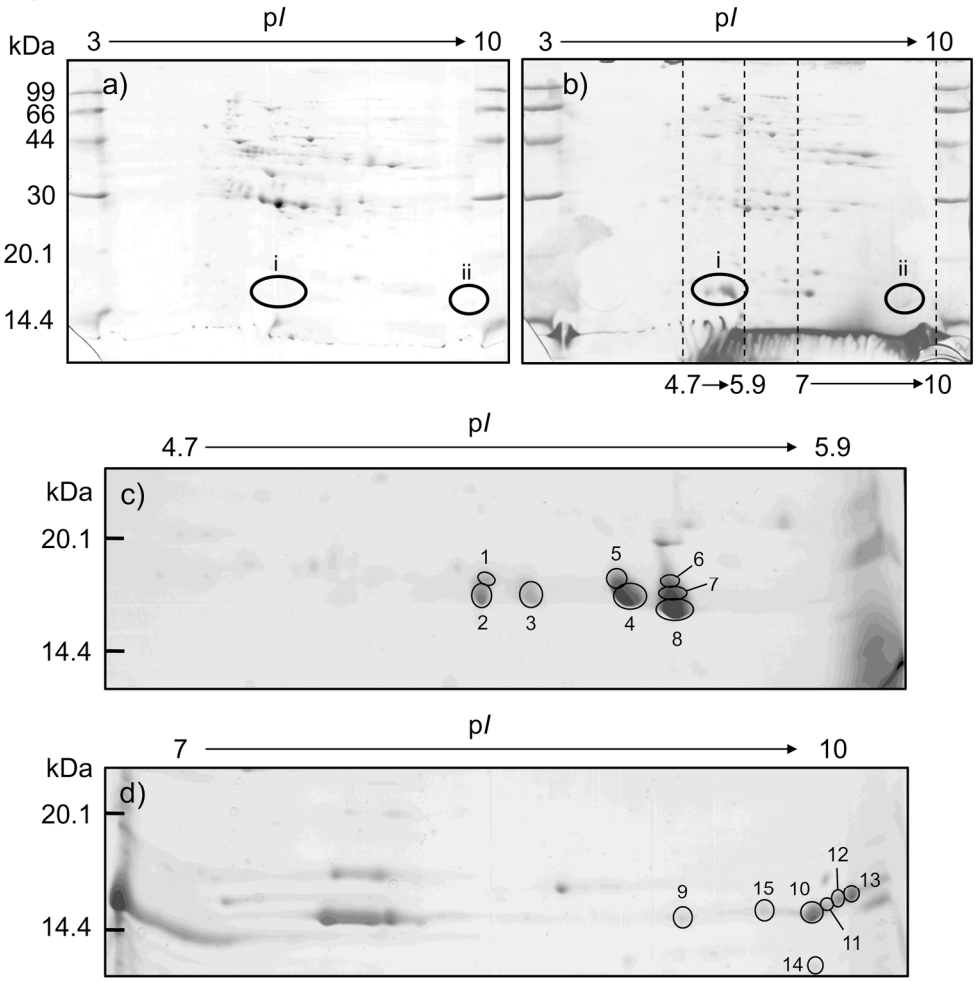

Figure 1
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Figure 2

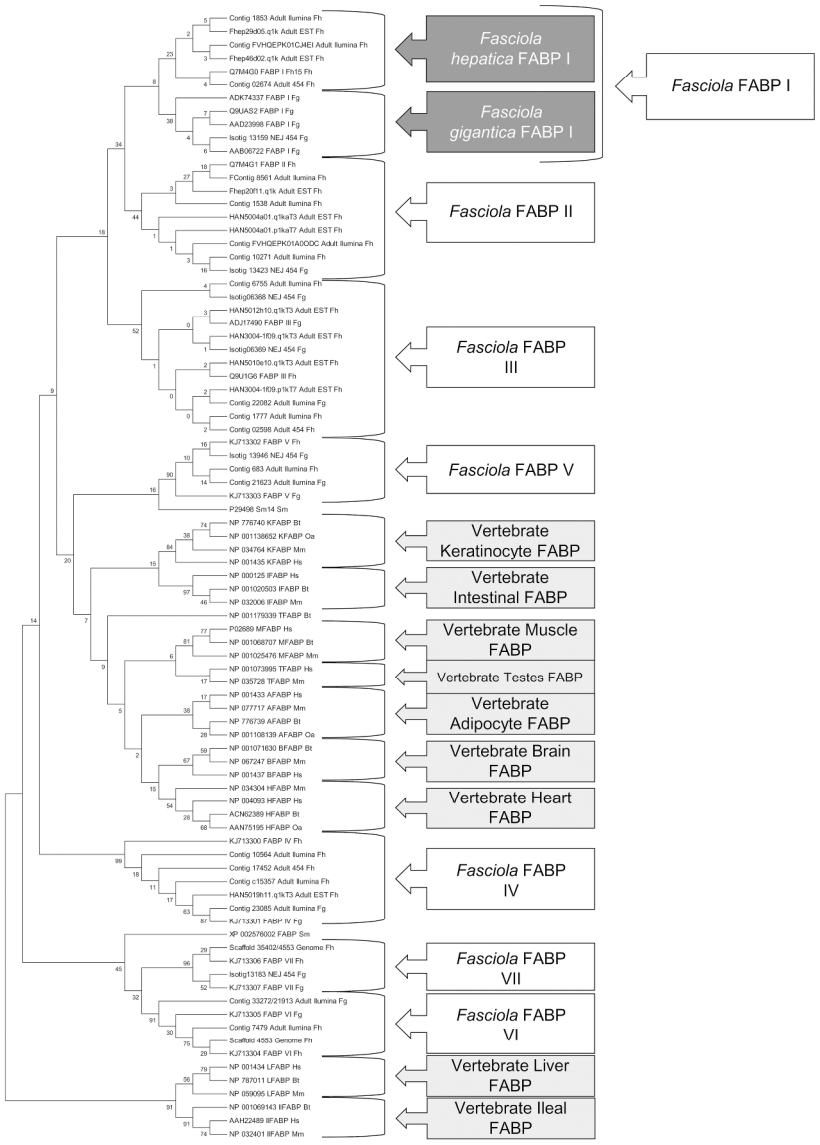

Figure 2

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Figure 3

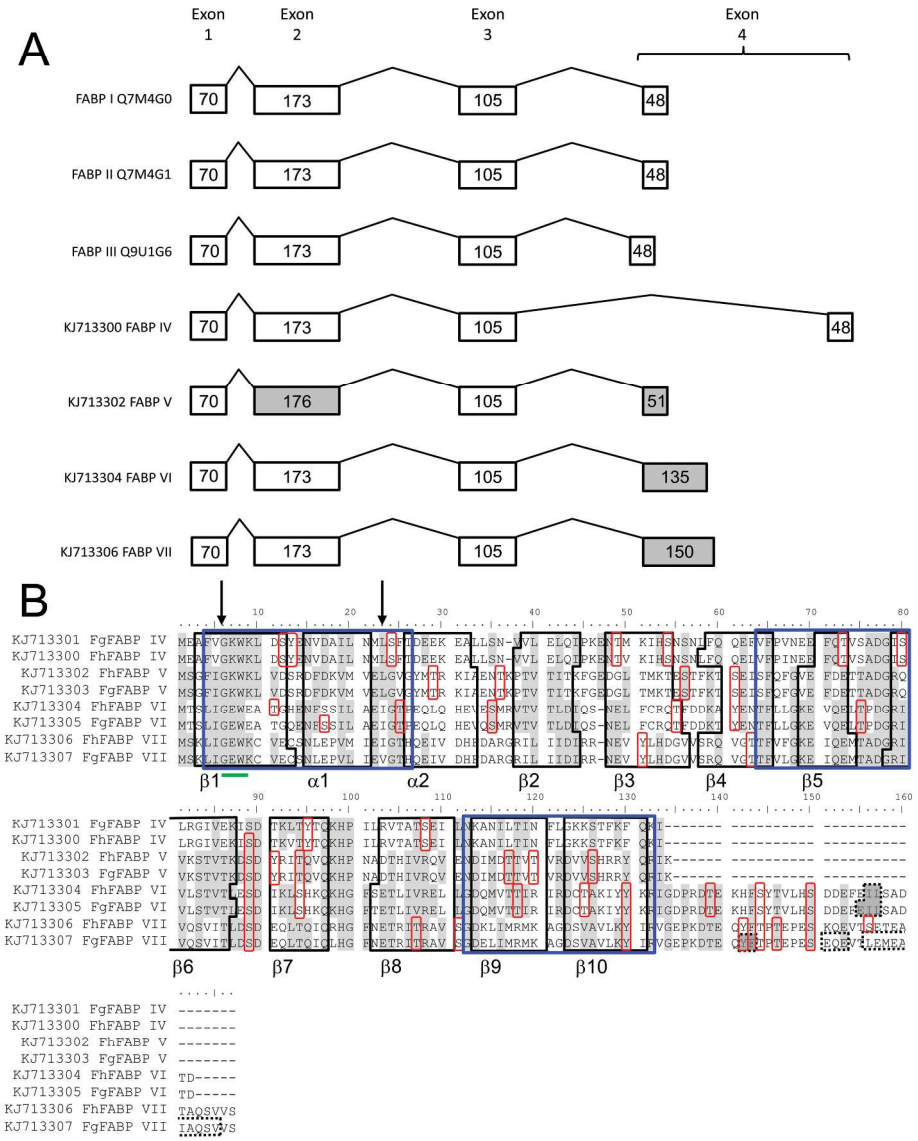

Figure 3

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Figure 4

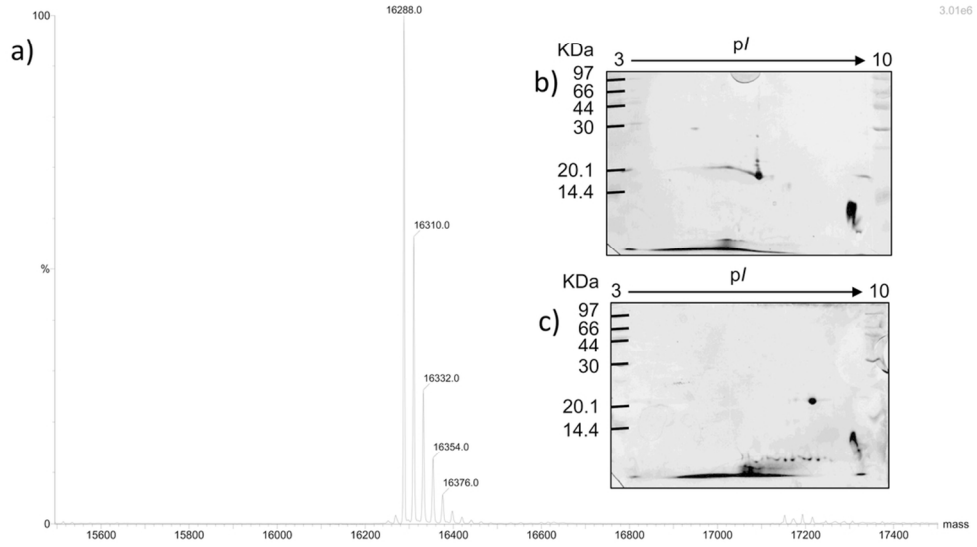

Figure 4

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Figure 5

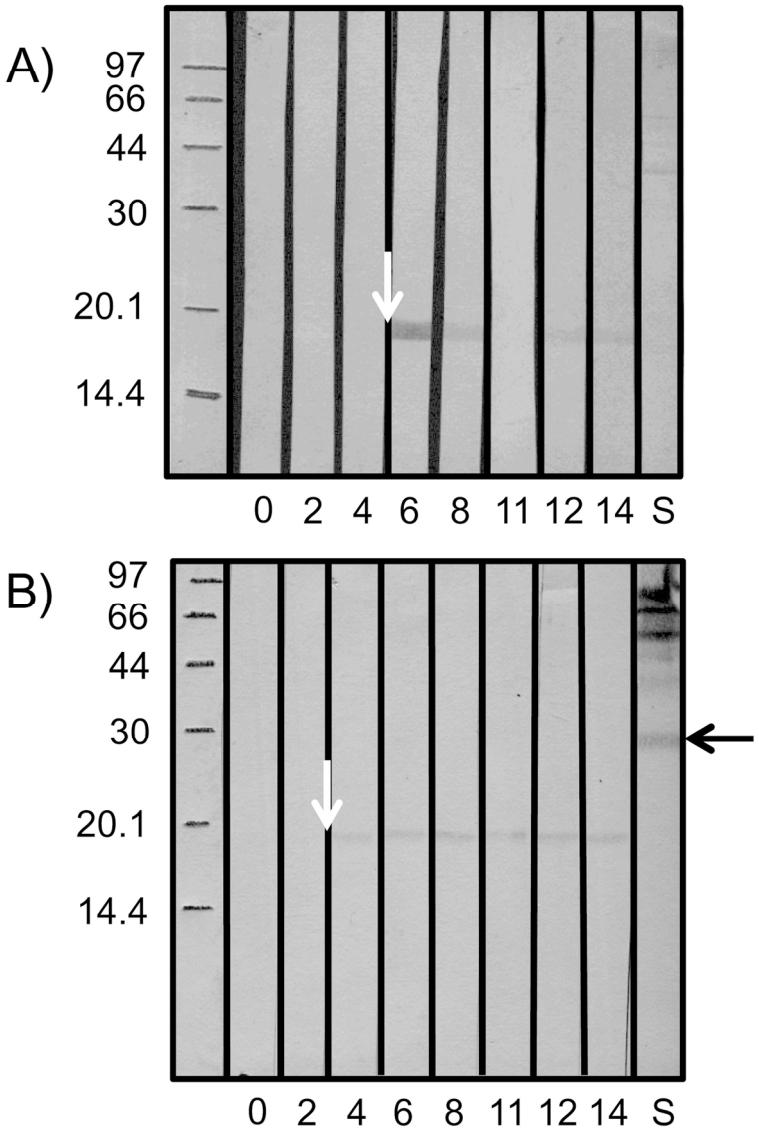

Figure 5

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