Author: L. McGonigle, A. Mousley, N. Marks, G. Brennan, J. Dalton, T. Spithill, T. Day and A. Maule
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Rapid Communication

The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduces gut penetration

Louise McGonigle [a], Angela Mousley [a], Nikki J. Marks [a], Gerard P. Brennan [a], John P. Dalton [b], Terry W. Spithill [c,d], Tim A. Day [e], Aaron G. Maule [a*]

[a] *Biomolecular Processes/Parasitology, School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, UK.*

[b] *Institute for the Biotechnology of Infectious Diseases, University of Technology, Sydney, Level 5, Block 4, Corner of Thomas and Harris Street, Ultimo, Sydney, NSW 2007, Australia*

[c] *Institute of Parasitology and FQRNT Centre for Host-Parasite Interactions, McGill University, 21,111 Lakeshore Rd, Ste-Anne de Bellevue, QC, Canada H9X 3V9*

[d] *School of Agricultural and Veterinary Sciences, Charles Sturt University, Wagga Wagga, Australia 2678*

[e] *Department of Biomedical Sciences and Neuroscience Program, Iowa State University, Ames IA 50011, USA*

[*] Corresponding author: Tel.: +44 (0)28 9097 2059; fax: +44 (0)28 9097 5877.

Email address: a.maule@qub.ac.uk (A.G. Maule).

Abstract

Probing protein function in parasitic flatworms is hampered by the difficulties associated with the development of transgenic approaches. Although RNA interference (RNAi) in

schistosomes shows much promise, it has not been reported in other trematodes. Here we show the successful silencing of the cysteine proteases cathepsin B and L in the infective stage of *Fasciola hepatica*, newly excysted juveniles (NEJs). Silencing resulted in marked reductions in target transcript levels and significant diminution in the encoded proteins in the gut. RNAi of either enzyme in NEJs induced transient, abnormal locomotory phenotypes, and significantly reduced penetration of the rat intestinal wall.

Keywords: Fasciola; newly excysted juvenile; cathepsin L; cathepsin B; RNA interference; tissue penetration

The expanding genomic and expressed sequence tag (EST) datasets for parasitic flatworms have helped highlight the need for technologies to aid their exploitation. Until recently, flatworm parasites have proven refractory to genetic manipulation and the absence of a tractable and appropriate model species, such as that provided for nematodes by *C. elegans*, has repressed scientific progress associated with the elucidation of gene function. RNA interference (RNAi), whereby double stranded (ds)RNA induces an intracellular cascade that results in degradation or suppression of the expression of homologous mRNA transcripts, is showing promise as a tool for exploiting the transcriptomic data from flatworm parasites. Such progress will aid the generation of biological data that could be used to inform control options. To date, the successful application of RNAi to trematodes has only been reported in *Schistosoma mansoni* and *Schistosoma japonicum* (Boyle et al., 2003; Skelly et al., 2003; Cheng et al., 2005).

Fasciolosis imposes an immense economic burden on global agriculture that is estimated to be over $2 billion per annum, and is a recognized food-borne zoonotic disease affecting approximately 2.4 million people (see McManus and Dalton, 2006). The causative agents, *Fasciola hepatica* and *Fasciola gigantica* have widespread distributions across several continents. The flukicide, triclabendazole is active against both adult and juvenile flukes and provides the front-line defence against fasciolosis in Europe and Australia. Unfortunately, triclabendazole-resistance is spreading in Europe and Australia and is beginning to compromise control efforts (see Brennan et al., 2007) emphasizing the need for an improved understanding of fluke biology that will support new drug/vaccine target discovery and validation processes. While to-date there is only a modest dataset of ~12K ESTs generated for *F. hepatica* (http://www.sanger.ac.uk/Projects/Helminths) this database is growing and will require the

methods to effectively probe gene function in this organism. To address this gap we set out to develop and apply RNAi approaches to the investigation of gene function in the infective stage of the worm.

The cathepsin-like cysteine proteases are amongst the most highly expressed proteins in *F. hepatica* where they play pivotal roles in host tissue penetration and digestion (see Dalton et al., 2003). Cathepsins Ls (FheCL) form a major monophyletic lineage of fluke proteases and display much allelic variation with at least 18 cathepsin L sequences known from *Fasciola sp.* (see Irving et al., 2003; Dalton et al., 2006). Adult flukes secrete up to 1 µg FheCL proteases per hour (representing ~5% of total adult protein) and ~10% of *F. hepatica* ESTs encode FheCL proteases (see Dalton et al., 2006). *Fasciola* FheCL proteases cleave within proteins (endoproteases), are stable enzymes active over a broad pH (3.0 to 8.0) range and are ideal for both intracorporeal and extracorporeal proteolysis of host proteins, including haemoglobin. Indeed, accumulating evidence supports the central role of FheCL proteases in adult fluke digestion (Dalton et al., 2003, 2006). FheCLs are also expressed by the infective stage of the parasite, termed newly excysted juveniles (NEJs), which exit the cyst within the host gut before penetrating the intestinal wall and migrating through the liver to the bile ducts where adult worms reside. Within juvenile worms, FheCL proteases have been proposed to facilitate excystment, gut penetration and liver migration by efficiently degrading proteins associated with the extracellular matrix (Berasain et al., 1997).

Although not as abundant or diverse as cathepsin L, cathepsin B (FheCB) proteases are also key enzymes in processes associated with parasite migration and establishment in the host, as well as playing household intracellular functions (see Wilson et al., 1998; Dalton et al., 2006). Indeed, a FheCB (GenBank accession # U58000; designated FhCatB1) was suggested to be the major secreted protease in NEJs and critical in the early migratory phases of juvenile fluke while the FheCL proteases become most influential during the liver-based migratory stages and adulthood (Wilson et al., 1998; Irving et al., 2003; Dalton et al., 2003). FheCBs are endoproteases which possess an occluding loop containing two His residues that confers a dipeptidyl exoprotease activity on the enzyme (although one of these His residues is absent from fluke FheCB, the remaining His forms a salt-bridge to Asp22 that serves to maintain the occluding loop structure) (Law et al., 2003). At least six FheCB proteases are known from *F. hepatica* and three from the tropical liver fluke *F. gigantica*: Fgicat-B2 and Fgicat-B3 are expressed in the juvenile stages only whereas Fgicat-B1 is expressed in all the intra-mammalian stages, implying that cat-B2 and 3 are involved in juvenile-specific activities, possibly associated

with excystment, gut penetration and migration. There is 99% identity between cat-B2 and the major secreted FheCB1 from *F. hepatica* NEJs (Meemon et al., 2004).

We selected FheCL and FheCB for our RNAi experiments because of their key roles in the biology and virulence of both juvenile and adult worms. We first examined the effects of exposing adult fluke and NEJs to dsRNA designed to *F. hepatica* FheCL1 (GenBank accession # U62288). The 176 bp dsRNA construct comprised a region that is highly conserved across *F. hepatica* FheCLs. Since most fluke FheCLs display over 90% nucleotide identity across this region, successful silencing could impact the global FheCL product of flukes. Adult flukes were recovered from bovine livers at a local abattoir (ABP Abattoir, Lurgan), transported to the laboratory in mammal saline (0.9% NaCl, 0.1% glucose) and maintained in *Fasciola* saline (FS; Dulbecco's Modified Eagle's Medium (DMEM) [w/o $NaHPO_3$ and PO_4^{-3}] plus 0.5 ml/ml distilled water, 2.2 mM Ca [$C_2H_3O_2$], 2.7 mM $MgSO_4$, 61.1 mM glucose, 1 µM serotonin, 5 µg/ml gentamycin, 15 mM HEPES, pH 7.4) at 37°C with regular changes (every 4-6 h). Metacercariae (generated at Queen's University Belfast) were excysted by incubation in 0.5% sodium hypochlorite for 20 min prior to centrifugation (2000 *g* for 2 min), washing in distilled water and incubation in 0.5% sodium bicarbonate, 0.4% sodium chloride, 0.2% sodium tauroglycholate, 0.07% concentrated HCl and 0.006% L-cysteine for up to 3 h at 37^{o}C. Metacercariae were transferred to watch glasses and allowed to excyst for a further 2-3 h. Released juveniles were removed from the excystment medium and maintained in fresh FS at 37^{o}C. Experimental worms were exposed to 0.1 mg/ml FheCL1-dsRNA in FS for 4 h at 37^{o}C within 2 h of recovery from abattoir (adults) or excystment (NEJs). After treatment, specimens were washed in fresh FS prior to total RNA extraction from 50 mg tissue and reverse transcriptase (rt)-PCR analysis of 2 µg total RNA using the QIAGEN OneStep RT-PCR kit (see Fig. 1 legend for details). Following rt-PCR, no measurable change in the FheCL1 products was detected in adult fluke (not shown), but there was a marked (~80%) reduction in the levels of FheCL1 transcripts in NEJs (see Fig. 1A; 30 NEJs in each experimental treatment, $n \geq 5$); the levels of FheCB1 transcript were unaltered in these parasites suggesting a specific reduction in the abundance of FheCL transcripts. Time- and concentration-matched controls included worms soaked in FS and worms soaked in FS containing dsRNA homologous to part of a chloroplast specific ribosomal protein (CSRP) derived from tomato (*Lycopersicon esculentum*) cDNA (GenBank accession # AY568722). The latter did not show significant homology to any helminth protein during BLAST searches. Our success in silencing NEJ FheCL was further examined using immunocytochemistry (ICC) with a FheCL-specific antiserum (Collins et al., 2004). NEJs maintained in FS or CSRP-dsRNA displayed abundant FheCL-immunoreactivity in their gut (Fig.

1C-E). The levels of FITC-fluorescence were quantified in five randomly-selected specimens from each treatment group using a Leica SP2 confocal scanning laser microscope and the quantification facility in Leica confocal software. Although the levels of fluorescence recorded in the control dsRNA-treated group appeared lower than those in the FS control group, these differences were not significant (Fig. 1B; P=0.129). However, the levels of FheCL-fluorescence were significantly reduced compared to those recorded in both control groups, suggesting that NEJ gut FheCLs are being actively synthesised and released *in vitro*.

With the success in silencing FheCL in NEJs, we repeated the experimental format for FheCB and generated a dsRNA transcript (207 bp) specific to the most abundant FheCB in NEJs (GenBank accession # U58000). Following 4h incubation in FS, FheCB1-dsRNA in FS or CSRP-dsRNA in FS for 4 h at 37^{o}C, NEJs were washed and prepared for rt-PCR or ICC analysis as described (30 NEJs in each experimental treatment, $n \geq 4$); FheCB1-dsRNA was not tested on adult fluke. As with FheCL, FheCB-RNAi resulted in a profound diminution (~78%) in the amount of FheCB transcript detected by rt-PCR (Fig. 2A). In this case, the levels of FheCL transcript were not altered, confirming the specificity of the FheCB-silencing. ICC analysis employed a rabbit antiserum raised against native FhCatB1 and previously shown to bind NEJ FheCB (Kennedy et al., 2006). Abundant FheCB-immunostaining was localized to the gut of NEJs, consistent with previous observations that employed an anti-bovine FheCB antiserum (Creaney et al., 1996). As with FheCL, the analysis of five randomly-selected NEJs from each experimental group revealed significant differences in the levels of FheCB-immunofluorescence. The CSRP-dsRNA did not significantly diminish FheCB-immunofluorescence in NEJs compared to worms maintained in dsRNA-free FS (P=0.0914). However, FheCB1-dsRNA treated NEJs displayed significant reductions in the levels of FheCB-fluorescence detected (Fig. 2B-F). As with the FheCL-RNAi experiments, the marked reduction in FheCB-immunofluorescence following treatment with FheCB1-dsRNA indicates the *in vitro* synthesis and release of gut-stored FheCB by NEJs.

The NEJ intestine is believed to fulfil a largely secretory role with the dual absorptive/secretory function developing at approximately 10 days during liver maturation and is particularly evident in adult fluke (see Wilson et al., 1998; Dalton et al., 2006). FheCB has been reported as the predominant cathepsin protease released by NEJs, contrasting to the situation in adult fluke where FheCL is the main secretory enzyme (Wilson et al., 1998). The ICC data in this study indicate that both FheCL and FheCB are highly abundant in the NEJ gut. The reduction in immunofluorescence signal in NEJs following silencing of the encoding transcripts further confirms the specificity of the two antisera. Furthermore, the reported secretory role of

the gut in these non-feeding NEJs is supported by observations that the FheCB- and FheCL-fluorescence levels drop dramatically after RNAi. Numerous critical functions in parasitism have been assigned to *F. hepatica* FheCB and FheCL proteases including involvement in excystment, gut penetration, sloughing of the tegumental glycocalyx, liver migration, immune evasion and digestion (Wilson et al., 1998; Dalton et al., 2003). However, in juveniles, most hypotheses on cathepsin function relate to a role for FheCB in gut penetration and/or liver migration. To probe this function further, we examined the impact that silencing FheCLs or FheCBs would have on the ability of NEJs to penetrate rat intestine (see Burden et al., 1981). Experimental design mirrored those described for the previous RNAi experiments with two control groups (FS and CSRP-dsRNA in FS) and dsRNA concentrations and exposure times being fixed at 0.1 mg/ml and 4 h, respectively. All experiments were repeated a minimum of three times and comprised placing 30 NEJs, immediately post-treatment (in FS or dsRNA) into rat gut sacks that were clamped and ligated at either end. In controls, the majority of NEJs had migrated through the mucosal wall within 30 min, but the experiment was allowed to proceed for 2 h with half-hourly monitoring to ensure recovery of NEJs. NEJs maintained in FS or exposed to control CSRP-dsRNA displayed 65 and 59% gut migration, respectively. However, NEJs exposed to FheCL1- or FheCB1-dsRNA displayed highly significant reduction in their ability to penetrate the gut sack (Fig. 3). These data suggest that both enzymes are important for gut penetration by NEJs and that silencing of either enzyme dramatically impacts this behaviour. The involvement of NEJ FheCL- and FheCB-proteases in gut penetration would seem consistent with their potential complementary actions as endo- and exo-proteases, making them ideally suited to a role in cleaving extracellular matrix proteins and penetrating the gut epithelia during migration. Comparison of the efficiency of gut penetration by FheCL- versus FheCB-silenced NEJs revealed a significantly greater reduction in penetration following FheCL-RNAi (see Fig. 3). This is noteworthy as previously published studies report the predominance of NEJ FheCB over NEJ FheCL in early migratory behaviours (Wilson et al., 1998; Law et al., 2003). Nevertheless, the data presented here emphasise the importance of both enzymes to early migratory activities within the host.

Visual observations of NEJs treated with dsRNA for either FheCB1 or FheCL1 revealed phenotypic differences associated with altered movement. Normal/control NEJs displayed locomotion that comprised a narrowing and stretching of the oral cone that is likely to be associated with circular muscle contraction, attachment of the acetabula to the surface, and then a shortening, consistent with the contraction of longitudinal muscles and relaxation of circular muscles. Immediately following FheCL-RNAi (4 h), most NEJs appeared paralysed; the

paralysis was reversible and normal movement was re-established within an hour in FS. Also, following FheCB-RNAi (4 h), NEJs displayed a variety of phenotypes including normal movement, erratic locomotion and paralysis. If these phenotypes occurred *ex vivo*, they may also have diminished the ability of worms to penetrate the gut effectively and could have contributed to the reduced gut penetration observed in this study. Additional experiments on the consequences of extended incubation in dsRNA revealed that 100% NEJs (20 worms, n=2) had stopped moving after 8 h (FheCL-RNAi) and 9 h (FheCB-RNAi) and had died after 12 h (FheCL-RNAi) and 14 h (FheCB-RNAi) in FS containing 0.1 mg/ml dsRNA; worm viability was identified as a failure to respond to a physical stimulus and vital stain (0.5% Evans' blue) uptake by tegument and somatic cells. In contrast, seventy five percent of worms exposed to CSRP-dsRNA were still moving after 14 h. A cathepsin B protease (SmCB1) was previously silenced in *S. mansoni* with different groups reporting either no phenotypic changes at 24 h and 40 days post-silencing (Skelly et al., 2003; Krautz-Peterson et al., 2007), or gross phenotypic changes in the form of stunted growth at 25 and 30 days post-dsRNA treatment *in vitro* or at 21 days in mice (Correnti et al., 2005). This disparity in the severity of the cathepsin-RNAi phenotypes reported for *S. mansoni* and *F. hepatica* further emphasises the pivotal importance of cysteine proteases to liver fluke biology and underscores their potential as targets for novel chemotherapeutics or vaccines (see Dalton et al., 2003; Law et al., 2003).

This study has successfully applied RNAi to silence two major secreted proteins of *F. hepatica* NEJs. Although some successes have been reported with RNAi in various life cycle stages of the blood fluke *S. mansoni* (Boyle et al., 2003; Dinguirard et al., 2006), adults required extended periods in culture or more complex dsRNA delivery methods including electroporation (Correnti et al., 2005; Krautz-Peterson et al., 2007), particle bombardment (Osman et al., 2006) or liposomal delivery (Skelly et al., 2003; Krautz-Peterson et al., 2007). Indeed, in schistosomules, silencing of cathepsin B was enhanced 100 to 1000 fold following square-wave electroporation compared to soaking methods (Krautz-Peterson et al., 2007); a similar approach may be needed to induce effective silencing in adult liver fluke. In the present study, silencing was induced by a simple soaking procedure which triggered dramatic effects that were detectable immediately following a 4 h exposure to either FheCL1-dsRNA or FheCB1-dsRNA; this method was ineffective in adult fluke. The specific and dramatic effects of FheCB/FheCL-RNAi on NEJs were evident from three parameters including, mRNA transcript levels, target protein expression and, functionally, by altered NEJ gut penetration. The data indicate that both FheCB and FheCL proteases are critical to NEJ penetration of the gut and that interference with the function of either has severe impact on worm virulence. The sensitivity of NEJs to RNAi

provides broad ranging opportunities to further probe the biology of cysteine proteases and other target genes in *F. hepatica*.

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Figure Legends

Fig. 1. The effects of RNA interference (RNAi) on the expression of cathepsin L (FheCL) in *Fasciola hepatica* newly excysted juveniles (NEJs). (A) Representative reverse-transcriptase PCR results showing a marked (~80%) reduction in FheCL transcript levels (lane 2) following treatment with double-stranded (ds)RNA for FheCL (Lanes: 1, *Fasciola* saline [FS] control; 2, FheCL1-dsRNA treated; 3, control-dsRNA treated; 4, no template control). Transcript levels of cathepsin B (FheCB) are unaffected (Lanes: 5, FS control; 6, FheCL1-dsRNA treated; 7, control-dsRNA treated; 8, no template control; M, marker lanes). (B-E) Data showing FheCL-immunoreactivity in NEJs exposed to the various treatment regimes. (B-D) Representative confocal scanning laser micrographs showing FheCL-fluorescence (green) in NEJs following a 4h soak in (B) FS, (C) 0.1 mg/ml FheCL1-dsRNA or (D) 0.1 mg/ml control-dsRNA. All scale bars represent 25 µm. (E) Barchart showing the levels of FITC fluorescence detected (using Leica confocal quantification software) in NEJs exposed to the different treatment regimes. Students *t* test revealed that FheCL-immunofluorescence was significantly ($P<0.0001$, ***; $P=0.0313$, *) diminished in NEJs treated with FheCL1-dsRNA. The FITC signal was quantified from five randomly-selected specimens from each treatment group and the data are presented as mean±SD in arbitrary fluorescence units. *Associated methods:* (A) FheCL1-dsRNA (176 bp) was generated from sequence-verified cDNA templates using T7-labelled (lower case) oligonucleotide primers designed to FheCL1 (upper case) (sense, $^{5`}$taatacgactcactatagggTTGTAGCCGTCCTTGGGGAAATA$^{3`}$; antisense, $^{5`}$taatacgactcactatagggGTATAGAAGCCAGTCACTTTGGC$^{3`}$) (GenBank accession # U62288). Megascript RNAi kits (Ambion) were used to convert purified T7 labelled DNA templates to single stranded RNA of sense and antisense polarity, as outlined in the manufacturer's instructions. Equal amounts of single stranded RNA were mixed and incubated at 68ºC for 10 min followed by incubation for 30 min at 37ºC. Following treatment with DNase, the purity and concentration of each dsRNA construct was determined using a NanoDrop ND1000-A spectrophotometer (Labtech UK) and aliquots of all dsRNAs were visualised on a 1.2% agarose gel to confirm integrity. Freshly excysted NEJs were soaked in 0.1 mg/ml dsRNA for 4 h at 37 ^{o}C in FS. Control-dsRNA (199 bp) was generated to a chloroplast specific ribosomal protein (CSRP) derived from tomato cDNA (GenBank accession # AY568722) using gene specific primers (upper case) flanked by T7 (lower case) polymerase sites (sense, $^{5`}$taatacgactcactatagggTGGCGACTCTTTCCCTTTC$^{3`}$; antisense, $^{5`}$taatacgactcactatagggCTATTCCTGACAGGAACA$^{3`}$) as for FheCL1-dsRNA. Following dsRNA

exposure, the RNAqueous kit (Ambion) was used to extract total RNA from each treatment group ($n \geq 5$) of 30 NEJs. 20 µg total RNA was then used to synthesise cDNA and PCR amplify specific gene products under normalized conditions and using the QIAGEN OneStep RT-PCR kit. All extracted RNA was DNAse treated prior to reverse transcription and PCR amplification. Gene specific primers (sense, $^{5`}$CATTCTCTGAGCAACAACTGGTC$^{3`}$; antisense, $^{5`}$GTATAGAAGCCAGTCACTTTGGC$^{3`}$) at a final concentration of 0.6 µM were used in 50 µl PCR reactions along with 27 µl of sterile water and 5 µl of template RNA. The following cycling conditions were used: 50°C for 30 min; 95°C for 15 min; 35 x 1 min cycles of 94°C, 58°C and 72°C; a final 10 min extension at 72°. RT-PCR products were visualised on 1.2% agarose gels containing 0.005% ethidium bromide (10 mg/ml) and images recorded using a Kodak Image Station 440; PCR products were quantified using Labworks Image Aquisition and Analysis Software version 4.6.00.0 (UVP Ltd. Cambridge UK). (B-D) Specimens were immunostained using the indirect method following a 4h fixation in 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS) [PBS, pH 7.4: 5 M NaCl, 0.025 M $NaH_2PO_4.2H_2O$, 0.075 M Na_2HPO_4] and then washed in antibody diluent (AbD: 0.1M PBS, pH 7.4 containing 0.1% [v/v] Triton X-100, 0.1% [w/v] bovine serum albumin [BSA], and 0.1% [w/v] NaN_3). The specimens were then incubated with anti-cathepsin L antiserum (raised in rabbit against recombinant FheCL; Collins et al., 2004) at 1:500 overnight and then washed three times in AbD and incubated overnight in secondary antibody: fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit immunoglobulin (1:1000). Specimens were rewashed (3x) in AbD and incubated for 24h in 1 ml AbD containing 200 ηg/ml phalloidin-tetramethylrhodamine isothiocyanate (TRITC) to counter-stain muscle. Finally, specimens were washed (3x) in AbD and mounted in PBS:glycerol (1:9) containing 2.5% (w/v) 1,4 diazabicyclo-2.2.2.octane (Sigma) and examined using confocal scanning laser microscopy (CSLM) (Leica AOBS SP2). No immunostaining was observed in controls in which the primary antiserum was omitted.

Fig. 2. The effects of RNA interference (RNAi) on the expression of cathepsin B (FheCB) in *Fasciola hepatica* newly excysted juveniles (NEJs). (A) Representative reverse-transcriptase PCR results showing a marked (78%) reduction in FheCB transcript levels (lanes 2 and 3) following treatment with double-stranded (ds)RNA for FheCB (Lanes: 1, *Fasciola* saline [FS] control; 2 and 3, FheCB1-dsRNA treated; 4, control-dsRNA treated; 5, no template control). Transcript levels of cathepsin L (FheCL) were unaffected (Lanes: 6, FS control; 7, FheCL1-dsRNA treated; 8, control-dsRNA treated; 9, no template control; M, marker lanes). (B-E) Data showing FheCB-immunoreactivity in NEJs exposed to the various treatment regimes. (B-D)

Representative confocal scanning laser micrographs showing FheCB-immunofluorescence (green) in NEJs following a 4h soak in (B) FS, (C) 0.1 mg/ml FheCB1-dsRNA or (D) 0.1 mg/ml control-dsRNA. All scale bars represent 25 µm. (E) Barchart showing the levels of FITC fluorescence detected (using Leica confocal quantification software) in NEJs exposed to the different treatment regimes. Note that FheCB-immunofluorescence was significantly diminished in NEJs treated with FheCB1-dsRNA (P=0.0002, ***; P=0.0062, **). The FITC signal was quantified from five randomly-selected specimens from each treatment group and the data are presented as mean±SD in arbitrary fluorescence units. Student's *t* tests confirmed significant differences as indicated. *Associated methods*: (A) FheCB1-dsRNA was generated from sequence-verified cDNA templates using T7-labelled (lower case) oligonucleotide primers designed to the major NEJ cathepsin B (upper case) (sense, $^{5`}$taatacgactcactatagggGCACGTACTGTGGTCAGGGGTG$^{3`}$; antisense, $^{5`}$taatacgactcactatagggCTCTTGCGCAAGGAGGTGTCGG$^{3`}$) (GenBank accession # U58000). Control-dsRNA was generated to a tomato chloroplast specific ribosomal protein (CSRP) as described previously (see Fig. 1 legend). Purified FheCB1-dsRNA constructs were 207 bp (control dsRNA was 199 bp) and freshly excysted NEJs were soaked in 0.1 mg/ml dsRNA in FS for 4 h at 37 ^{o}C. Gene specific primers outside (sense) and inside (antisense) the FheCB1-dsRNA target (sense, $^{5`}$CGTTTGGCCGCAGCAGATCCAC$^{3`}$; antisense, $^{5`}$CTCTTGCGCAAGGAGGTGTCGG$^{3`}$) at a final concentration of 0.6 µM were used under normalized conditions in 50 µl PCR reactions with the following cycling conditions: 50°C for 30 min; 95°C for 15 min; 35 x 1 min cycles of 94°C, 60°C and 72°C; a final extension of 72° for 10 min (see Fig. 1 legend for other details). (B-D) Specimens were immunostained as described (Fig. 1 legend) with a primary antiserum raised in rabbits against *F. hepatica* FheCB-1 (Kennedy et al., 2006) at 1:500 (20 µg/ml) overnight prior to further processing. No immunostaining was observed in controls in which the primary antiserum was omitted.

Fig. 3. Barchart showing the effects of cathepsin RNA interference (RNAi) on the penetration of rat gut sacks by *Fasciola hepatica* newly excysted juveniles (NEJs). Note that 65±1.1% of Fasciola saline (FS)-treated NEJs completed the migration, i.e. were collected outside the gut sac. Treatment of NEJs with dsRNA (0.1 mg/ml in FS; 4 h at 37^{o}C) for either cathepsin B (CB) or cathepsin L (CL) resulted in a highly significant reduction in gut penetration compared to either FS-treated (P<0.0001 for CB or CL), or control-dsRNA (chloroplast specific ribosomal protein [CSRP], 0.1 mg/ml for 4h at 37^{o}C) treated (CB, P=0.004; CL, P=0.001) NEJs. Student's *t* test revealed that the effects of the FheCL1-dsRNA were significantly greater than the effects

of the FheCB1-dsRNA (P=0.018, *). Briefly, 30 NEJs from each experimental group were placed in freshly excised rat gut (10 cm comprising the duodenum) segments that were clamped and ligated at either end and maintained in FS for 2 h at 37^{o}C ($n \geq 3$). After this time, the number of NEJs that had penetrated the gut sack was counted and data are expressed as mean±SD percent gut penetration.