Fasciola hepatica antioxidant and protease-inhibitor cocktail recombinant vaccines administered five times elicit potent and sustained immune responses in sheep but do not confer protection

Krystyna Cwiklinski[1,2*], Amanda McEvoy[3], Jesús López Corrales[1], Heather Jewhurst[1], Nichola Eliza Davies Calvani[1], Carolina De Marco Verissimo[1], Amber Louise Dorey[1], Orla M. Keane[4], John Pius Dalton[1], Richard Lalor[1].

[1]Molecular Parasitology Laboratory, Centre for One Health and Ryan Institute, National University of Ireland Galway, Galway, Ireland.

[2]Current address: Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, UK

[3] Animal & Bioscience Department, Teagasc Mellows Campus, Athenry, Co., Galway, Ireland.

[4] Animal & Bioscience Department, Teagasc Grange, Dunsany, Co. Meath, Ireland.

* Corresponding author

E-mail: krystyna.cwiklinski@liverpool.ac.uk

Abstract

Our laboratory's vaccine development strategy against the livestock parasite *Fasciola hepatica* centres around disrupting key biological processes by combining groups of antigens with similar/complementary functional actions into a single vaccine cocktail. In this study the focus was on antioxidant protein vaccines and a protease inhibitor vaccine aimed at disrupting the parasite's ability to defend against oxidative stress and protease-inhibitor balance, respectively. Two combinations of recombinantly expressed antioxidants were assessed, namely peroxiredoxin (rFhPrx), thioredoxin (rFhTrx) and thioredoxin-glutathione reductase (rFhTGR) (Group 1) and rFhPrx, rFhTrx, and two superoxide dismutases (rFhSOD1 and rFhSOD3) (Group 2). The protease inhibitor vaccine cocktail included representatives of each of the key secreted protease inhibitor families, namely a Kunitz-type inhibitor (rFhKT1), a serpin (rFhSrp1) and a stefin, (rFhStf1) (Group 3). The vaccine combinations were formulated in adjuvant Montanide 61VG administered at five timepoints; two before experimental challenge with 60 *F. hepatica* metacercariae and three after infection. The vaccine combinations did not reduce the liver fluke burden, and only Group 2 displayed a marginal reduction in egg viability (8.2 %). Despite previous results showing an effect of liver fluke vaccines on overall weight gain in infected animals, no significant (P value >0.05) impact on weight gain was observed in this study. Antibodies were elicited against all the vaccine antigens within the cocktails and were maintained at high levels to the end of the trial, due to our strategy of continuing vaccine administration after infection. However, these responses were not boosted by the challenge *F. hepatica* infection. A comparative analysis with previous vaccine data using a protease inhibitor vaccine found no repeat of the promising outcomes associated with this vaccine, indicating that the addition of rFhSrp1 to the vaccine cocktail did not improve vaccine

efficacy. Assessment of liver pathology across the two trials using a modified liver enzyme score (glutamate dehydrogenase to platelet ratio) at eight weeks post infection suggests an association with liver fluke burden above 45 flukes, which could be used to predict liver pathology in future trials. The results reported in this study highlight the ambiguousness in liver fluke vaccine development and the difficulty in obtaining consistent and repeatable protection. This work stresses the need for repetition of trials and the use of sufficiently sized groups to assess vaccine efficacy with adequate statistical power.

Keywords: *Fasciola hepatica*; sheep; vaccines; antioxidants; protease-inhibitors

1. Introduction

There has been extensive debate over the past decade of how we can meet the challenges of a growing global population with the increased demand for animal products while maintaining animal productivity and welfare and minimising the impact on the environment (Hume et al., 2011). Critical to the maintenance of livestock productivity is the control of helminth parasites in an everchanging climate that is facilitating increased spread, prevalence and parasite survival (Morgan et al., 2013; Caminade et al., 2015; Charlier et al., 2020).

The liver fluke, *Fasciola hepatica*, infects a wide range of mammals but is most widely known for its negative impact on sheep and cattle production, which can be caused by a burden of as few as 30 parasites (Toet et al., 2014). The clinical presentations of fasciolosis (reviewed in Lalor et al., 2021) that occur following the ingestion of the infective stage parasite, the metacercariae, include ill-thrift and detrimental effects on growth rate, wool and milk production and fertility. It is also a significant cause of abattoir liver condemnation (MacGillivray et al., 2013; Mazeri et al., 2017; Nyirenda et al., 2019; Utrera-Quintana et al., 2022). In sheep, sudden death can follow the onset of severe acute disease from large numbers of ingested parasites, although sub-clinical infections, consisting of low fluke burdens which nevertheless impact animal productivity, are more common (Stuen and Ersdal, 2022).

Current liver fluke control is reliant on anthelminthics, but in light of the emergence of drug resistance a more strategic use of drugs dependent on the time of year and liver fluke forecasts is encouraged alongside grazing management (SCOPS a; COWS; Animal Health Ireland, 2021). Liver fluke vaccines are being evaluated to add to the

repertoire of control strategies, and despite the great effort given to the identification and manufacture of molecular vaccines, progress has been slow and inconsistent (Spithill et al., 2022). Our pursuit of novel anti-fluke sub-unit vaccines focuses on disrupting key parasite biological processes by combining several functionally-related molecules, for example, antioxidants and protease inhibitors, in vaccine cocktails.

The study by Cwiklinski et al. (2022) recently showed that disrupting the parasite's protease-antiprotease balance impacts liver fluke egg viability and allows greater weight gain in infected sheep. Here, the protease inhibitor vaccine cocktail was refined to incorporate representatives of the three main classes of protease inhibitors actively secreted by multiple *F. hepatica* life cycle stages, namely kunitz-type inhibitor 1 (FhKT1), serpin 1 (FhSrp1) and stefin1 (FhStf1). Each of these protease inhibitors has been shown to play an important role during the early stages of infection in regulating parasite and/or host protease activity and potentially suppressing the host immune response. They were selected based on their high levels of expression by the newly excysted juveniles (NEJ) and immature flukes (Smith et al., 2016; De Marco Verissimo et al., 2020; Smith et al., 2020; Cwiklinski et al., 2022; De Marco Verissimo et al., 2022); stages that cause the most pathology during infection.

The antioxidants peroxiredoxin (FhPrx) and thioredoxin-glutathione reductase (FhTGR) have been targeted in *F. hepatica* vaccine trials, either as single antigen vaccines or in combination with other non-related antigens, with varied success (Mendes et al., 2010; Maggioli et al., 2011b; Buffoni et al., 2012; Maggioli et al., 2016; Zafra et al., 2021). In this study the aim was to target multiple players of the *F. hepatica* antioxidant system, known to be important for defence against the early innate immune

response directed towards the NEJ and the increased levels of oxidative stress exerted on the migrating juveniles in the liver (Dorey et al., 2021; Calvani et al., 2022). Omics and biochemical studies highlight the expression of these key components of the thiol-dependent system by the NEJ, immature and adult flukes, namely FhPrx, thioredoxin (FhTrx) and FhTGR (Dorey et al., 2021), and the metalloenzyme antioxidants involved in defence against oxygen free radicals, the superoxide dismutases (SODs; FhSOD1, FhSOD3; Calvani et al., 2022).

In the present study, three *F. hepatica* vaccine combinations were evaluated, two cocktails of antioxidant proteins and one protease inhibitor protein cocktail in sheep experimentally infected with *F. hepatica* metacercariae. Vaccine efficacy was assessed as the impact on liver fluke burden and egg number and viability, in addition to overall markers of animal health. Lastly, the results of this study were compared to previously reported vaccine trials to discern the impact of liver fluke burden on animal health markers. The data shows that despite eliciting potent and sustained immune responses to our vaccine cocktails, these did not protect sheep against a single challenge of *F. hepatica* metacercariae.

2. Materials and methods

2.1 Ethical statement

Experimental procedures on animals received approval from the Teagasc Animal Ethics Committee (Ireland) (approval number TAEC2021-298) and were conducted under authorization from the Health Products Regulatory Authority (Dublin, Ireland; authorization number AE19132/P115) in accordance with the EU Directive 2010/63/EU.

2.2 Production of recombinant proteins

The eight *F. hepatica* gene sequences selected for this vaccine trial were confirmed by analysis of the *F. hepatica* genome (WormBase ParaSite: PRJEB6687 and PRJEB25283; Cwiklinski et al., 2015), and prepared for recombinant expression as previously described (Table 1).

Recombinant expression of the *F. hepatica* antigens was carried out using two expression systems. Three antigens (rFhCL1, rFhKT1, and rFhStf-1) were expressed with a C-terminal His-tag using methylotrophic yeast *Pichia pastoris* and purified using the protocol previously described (Collins et al., 2004; Smith et al., 2016; Cwiklinski et al., 2022). The remaining six antigens (rFhPrx, rFhSrp1, rFhSOD1, rFhSOD3, rFhTrx, rFhTGR) were expressed with a C-terminal His-tag using *Escherichia coli* and purified using the protocol described previously (De Marco Verissimo et al., 2020; Calvani et al., 2022). New pET-28a(+) vectors with a C-terminal His-tag (GenScript) were prepared for the antioxidant sequences (FhPrx, FhTrx, FhTGR) used in this study. The FhTGR sequence contained a selenocysteine to cysteine mutation at position 592, as previously described by Maggioli et al. (2011b).

Protein concentration and purity for all the expressed proteins were verified by Bradford Protein Assay (Bio-Rad) and by 4-20 % SDS-PAGE gels (Bio-Rad) stained with Biosafe Coomassie (Bio-Rad), respectively. The gels were visualised using a G:BOX Chemi XRQ imager (Syngene).

2.3 Vaccine trial design

Sixty crossbreed sheep, four-months of age (2 ewes, 7 rams, 51 wethers), reared in Co. Galway, Ireland on farms considered to be liver fluke-free, were purchased and housed at the Teagasc sheep research farm at Athenry, Co. Galway. All sheep were treated on arrival to the research farm with monepantel (Zolvix, Elanco Inc. 2.5 mg/kg) and vaccinated with Scabivax Forte (MSD Animal Health) and Heptavac P (MSD Animal Health). While housed, sheep were fed a diet of ad-lib grass silage with 0.4 kg of concentrates per day with free access to water. Three weeks post-housing all sheep were tested for liver fluke eggs by faecal egg count (FEC) and for anti-FhCL1 serum antibodies (López Corrales et al., 2021) and were considered liver fluke-free. Animals were subsequently allocated into four groups based on body weight: Group 1 (G1): vaccinated with vaccine 1 (rFhPrx, rFhTrx, rFhTGR) and infected (n=15, male), Group 2 (G2): vaccinated with vaccine 2 (rFhPrx, rFhSOD1, rFhSOD3, rFhTrx) and infected (n=15, male), Group 3 (G3): vaccinated with vaccine 3 (rFhKT1, rFhSrp1, rFhStf1) and infected (n=15, male) and Group 4 (G4): unvaccinated and infected (n=15; 13 male, 2 female). The number of animals per group was calculated by power analysis based on parasite burden at necropsy, assuming infection establishment rates in unvaccinated animals between 50-70 % and average reduction in parasite number between 20-40 % in vaccinated animals, as per our previous studies (Cwiklinski et al., 2022) and other published studies in the literature (Toet et al., 2014; Spithill et al., 2022). Ten days after the first vaccine administration sheep were turned out to liver fluke-free pasture where they were exposed to gastrointestinal nematodes (GIN). Sheep were re-housed from 6 weeks post-infection (WPI) and maintained indoors as described above until the end of the trial. Following exposure to pasture individual FEC for *Nematodirus* spp. other *Trichostrongylus* spp. and *Strongyloides papillous* were performed on individual samples, followed by pooled monitoring using the

FECPAK method (further details provided in Supplementary Table S1). The animals were treated according to these results; they remained infected with GIN until the end of the trial (Supplementary Table S1).

The vaccinated animals (G1-G3) received five subcutaneous injections, each comprised of a total protein amount of 150 µg, made up of 50 µg of each antigen except for the SOD proteins, which were each administered as 25 µg, mixed in Montanide 61VG (Seppic; ratio of adjuvant: antigen, 60:40) to a final volume of 2 ml. The first two vaccinations were administered three weeks apart, followed by oral infection with 60 visually viable *F. hepatica* metacercariae (Italian isolate; Ridgeway Research Ltd) in 10 ml water using a 10 ml syringe lacking a needle four weeks after the second vaccination. Each animal was monitored for coughing/expelling the water directly after the infection dose was administered. The remaining three vaccinations were administered at four WPI and at eight-week intervals thereafter.

Body weight was assessed throughout the trial every three to four weeks, ten timepoints in total (Weeks 0, 3, 7, 11, 15, 19, 23, 27, 31 and 34). FEC were carried out within two weeks of sample collection (5 g, stored at 4 °C until processing) at 18, 22 and 26 WPI using standard sedimentation protocols for the detection of *F. hepatica* eggs as previously described (López Corrales et al., 2021). Animals were euthanised at 27 WPI by captive blot, followed by immediate exsanguination. At necropsy, the liver and gall bladder were recovered and photographed for assessment of gross pathology, and total enumeration of fluke burden and parasite eggs was carried out.

2.4 Egg hatch assay (EHA)

Eggs recovered from the gall bladder from each animal were enumerated and the hatch rate was evaluated using *F. hepatica* egg hatch assay protocols previously described (Fairweather et al., 2012; Cwiklinski et al., 2022). Briefly, approximately 100 eggs from each animal were placed in duplicate in a 12-well plate in 1 ml of tap water and embryonated in the dark at 26 °C for 14 days. Embryonation assessment was carried out after exposure to light for 30 min. Egg hatching was stimulated by an overnight incubation at 4 °C, followed by a 30 min incubation at 26 °C and exposure to light. Egg development was determined using the protocol by Fairweather et al. (2012).

2.5 Analysis of anti-*F. hepatica* antigen specific antibodies (total IgG) in sheep sera samples by ELISA

Flat-bottom 96 well microtitre plates (NuncTM Maxisorp TM, Thermo Fisher Scientific) were coated with 100 µl of 5 µg/ml of the individual recombinant antigens (based on coating saturation ELISA to determine the optimal concentration), or positive control (rFhCL1) in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After five washes with 100 µl of PBS-0.05 % Tween 20 (PBST), 100 µl/well of blocking buffer (2 % bovine serum albumin diluted in PBST) was added and the plates incubated for 1 h at 37 °C, followed by five washes with PBST. Serial dilution of the sera samples and the secondary antibody was carried out to determine the optimal dilution to be used in the assay. Sheep sera samples were diluted in serum dilution buffer (PBS, 0.5 % Tween-80, 0.5 M NaCl) at 1: 10,000. One hundred µl of the diluted serum samples were added in triplicate to the plates and incubated for 1 h at 37 °C. After washing five times, 100 µl/well of HRP-conjugated donkey anti-sheep IgG (Thermo Fisher Scientific) diluted in blocking buffer (1: 25,000) was added and the plates were

incubated for 1 h at 37 °C. Following five washes, 100 µl/well of 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich) was added and the plates incubated at room temperature for 5.5 min. The reaction was stopped by the addition of 100 µl/well of 1 M sulphuric acid (Sigma-Aldrich) and the optical density was determined at a wavelength of 450 nm (OD450) in a PolarStar Omega spectrophotometer (BMG Labtech).

2.6 Haematological analysis and liver enzyme assays

Blood samples were collected for haematological analysis and biochemical analysis. Serum liver enzyme levels (glutamate dehydrogenase, GLDH, and gamma glutamyl transferase, GGT) were determined using the UV (DGKC; Randox Laboratories Ltd) and Szasz (Glenbio Ltd) methods, respectively, and analysed on an AU480 clinical chemistry analyser (Beckman Coulter Inc), at six time-points during the trial: (a) Week 0; at the beginning of the trial, (b) Week 3; post-primary vaccination, (c) Week 7; pre challenge (0 WPI), (d) Week 15; 8 WPI, post three vaccinations, (e) Week 27; 20 WPI and (f) Week 33; 26 WPI, at necropsy. Haematological analysis was performed using an Advia 2120 haematology analyser and included red blood cell counts, platelet counts, total haemoglobin, and differential white blood cell count defined as a percentage of the total cell count for lymphocyte, neutrophil, eosinophil and monocyte subsets.

Liver enzyme to platelet ratios were calculated using the formula derived for the aspartate aminotransferase (AST) to platelet ratio index (APRI) developed by Wai et al. (2003).

$$\frac{\text{Liver enzyme/upper normal value}}{\text{Platelet count } (10^9/\text{L})} \times 100$$

The upper normal values in sheep for glutamate dehydrogenase (GLDH; iu/L) and gamma glutamyl transferase (GGT; iu/L) used in the calculations were 25 and 50, respectively (University of Guelph; Jackson and Cockcroft, 2002).

2.7 Comparative analysis with previous *F. hepatica* vaccine trials

The vaccine cocktail used by Cwiklinski et al. (2022) contained three recombinant stefins (rFhStf-1, rFhStf-2, rFhStf-3) and a Kunitz type inhibitor (rFhKT1) formulated in adjuvant Montanide 61VG, which was administered three times by sub-cutaneous injection prior to infection with *F. hepatica* metacercariae (Trial 2: Italian isolate, Ridgeway Research Ltd). Data from the haematological and biochemical analyses generated in this previous study were compared with the corresponding data from this trial, and graphically represented using GraphPad Prism (v5. 03).

2.8 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (v5.03). Group comparisons were assessed for normal distribution using the D'Agostino and Pearson omnibus normality test and the Shapiro Wilk normality test. All data was analysed using One-way ANOVA with Tukey's Multiple comparisons, with the exception of the GLDH data that was analysed as non-parametric data using the Kruskall-Wallis Test with Dunn's multiple comparisons test. P values of <0.05 were considered significant.

3. Results

3.1 Recombinant production of the *F. hepatica* vaccine antigens

Recombinant protein production was carried out using two approaches, a bacterial (*E. coli*) and a yeast (*P. pastoris)* expression system. Independent of the expression

system, all the recombinant proteins were produced with a C-terminal six-His-tag for purification by one-step Nickle-chelate affinity chromatography, which resulted in proteins of high yield and purity. All the proteins were analysed by SDS-PAGE, which showed bands at the expected molecular sizes (Fig.1; Table 1).

3.2 Assessment of liver fluke burden by enumeration of adult flukes and eggs

At necropsy total adult liver fluke enumeration was carried out to determine the parasite burden in the vaccinated animals compared to the *F. hepatica* infected control group (Table 2). Across the four groups a wide range of 4-67 adult flukes were recovered, reflecting a parasite take of the original infective dose of between ~7 % and 100 %. The infective metacercarial dose was prepared to ensure 60 viable parasites on visible inspection; therefore, in the case of the animal with 67 adult flukes, more viable metacercariae were administered than intended. No protection was observed within the vaccinated groups (G1-G3) compared to the control group (G4), with no significant difference observed between the groups.

FEC were carried out at three timepoints after patency had been reached, namely 18, 22 and 26 WPI (Table 3). No significant differences were observed between the vaccinated groups compared to the control group; all groups shed comparable numbers of eggs, which increased as the infections progressed.

Enumeration of the eggs recovered from the gall bladder found that more eggs were recovered from the vaccine groups than the control group (Table 4). Assessment of egg viability using the egg hatch assay showed no inhibitory effect was observed for vaccine groups 1 (G1) and 3 (G3) compared with the control group (G4); however, a

marginal reduction in egg viability of 8.7 % was observed for vaccine group 2 (G2) compared with the control group 4.

3.3 Antibody responses

Typically, *F. hepatica* vaccine trials employ a strategy of three vaccine doses before the animals are experimentally or naturally infected. In our previous vaccine trials, antibody responses to the vaccine antigens were observed to wane over the course of the experimental trial, indicating a lack of boosting from infection with *F. hepatica*. Therefore, in this study, additional vaccine doses were used to assess whether they would prolong the antibody responses to enhance our vaccine efficacy. Five vaccine doses were administered, two of which were given prior to challenge with *F. hepatica* metacercariae. The remaining three doses were administered following infection at key developmental time-points for the parasite, namely during the early liver migratory stages (4 WPI), when the parasites reached patency (12 WPI) and after establishment within the bile ducts (20 WPI).

Strong IgG antibody responses were induced by the vaccination protocol for all the vaccine antigens in each of the three vaccine cocktails (Fig.2), apart from rFhTGR in G1 (Fig. 2A) from which lower OD values were observed than for rFhCL1. For each of the vaccine antigens, the antibody responses were sustained as the trial progressed, and for the majority of the antigens were observed at the highest mean values at the end of the trial. Importantly, however, analysis of the natural response during *F. hepatica* infection in those animals not vaccinated, highlighted that the majority of the vaccine antigens used in this study do not elicit a significant antibody response and are thus considered not immunogenic during natural infection (Fig.2D). Antibody

responses were observed for rFhStf1 and rFhKT1 following 20 WPI but these were low relative to those observed for rFhCL1, which has been shown to be a major *F. hepatica* immunogen (López Corrales et al., 2021).

3.4 Impact of liver fluke on weight gain and levels of haemoglobin

It has been previously shown that *F. hepatica* vaccines can result in a positive effect on weight gain over the course of a vaccine trial and are associated with increased levels of haemoglobin indicative of a reduction in the risk of blood loss and anaemia (Cwiklinski et al., 2022). In this study, the average weight of the sheep at the beginning of the vaccine trial was 42.7 kg. However, no significant differences in weight gain by the end of trial were observed between the vaccinated animals and the control group (Fig.3; G1: 45.3 kg; G2: 47.3 kg; G3: 45.5 kg; G4: 48.2 kg; P value >0.05). Analysis of the daily weight gain (DWG) calculated at four-week intervals further shows that all the animals in the study displayed a similar weight gain profile irrespective of their vaccination status (Supplementary Fig. S1).

No significant differences were observed in the levels of haemoglobin between the four groups (P value >0.05). At 8 WPI haemoglobin levels are within the typical normal reference range (10 - 15 g/dL), despite harbouring liver migrating parasites that can cause extensive damage that can lead to haemorrhaging. By 20 WPI, consistent with the adult parasites extensively blood feeding, the haemoglobin levels were trending below the lower limit in all four groups.

3.5 Assessment of liver pathology

Fasciolosis is associated with increased levels of liver enzymes, including glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (GGT), detectable within serum reflecting the liver damage caused by the migrating parasites (reviewed Lalor et al., 2021). While the levels of these enzymes increase as the disease progresses, they are often not reflective of parasite burden and resulting pathology (Cwiklinski et al., 2022).

In human health, biomarkers can be used to estimate liver fibrosis and liver dysfunction, including combinations of markers that can improve sensitivity and specificity of the tests, such as the aspartate aminotransferase (AST) to platelet ratio index (APRI) developed by Wai et al. (2003). In this study we used a similar approach by calculating the ratio of GLDH and GGT to the platelet count at three timepoints during the trial (8 WPI, 20 WPI and 26 WPI) and correlating this value to the adult liver burden and levels of haemoglobin (Fig. 4; Supplementary Fig. S2 and S3). Based on the APRI scoring of 0.5 denoting minimal to no liver damage, a similar cut-off for the GLDH and GGT enzymes was used, with scores greater than 0.5 indicative of liver pathology.

No correlations between the number of adult fluke and liver enzyme to PLT ratio scores were observed at any timepoint and there were no differences depending on the vaccination status of the animals. Similarly, no correlations were observed for the haemoglobin data at the respective time-points. However, comparative analyses across the three timepoints revealed that the highest number of values above 0.5 were observed for the GLDH PLT ratio comparisons at 8 WPI, indicating a high level of pathology was being caused at this timepoint when the parasites are migrating within

the liver (Fig. 4; Supplementary Fig. S2). A similar trend was not observed for GGT, which was understandable given that GGT is a marker of damage to the bile ducts.

Assessment of gross liver pathology at necropsy revealed low levels of pathology (Supplementary Fig. S4A), predominantly associated with the left lobe, with examples of bile duct hyperplasia and fibrosis typically seen during *F. hepatica* infection (Sangster et al., 2022). The liver pathology scores showed moderate, but significant, positive correlation with the number of adult flukes recovered at necropsy ($r = 0.4370$; $p < 0.001$; Supplementary Fig. S4B). While no correlation was observed between the gross pathology scores and the liver enzyme to platelet ratios, the low GLDH/GGT PLT ratio scores reflected the overall low level of gross pathology observed in these animals.

3.7 Effect of liver fluke burden on parameters associated with fasciolosis – comparative analyses between *F. hepatica* vaccine trials

Haematological and biochemical data from the comparative groups from our current study were compared with the data from Cwiklinski et al. (2022), namely control *F. hepatica* infected sheep and sheep vaccinated with the protease inhibitor cocktail. One of the differences between these trials was the parasite dose given, here 60 parasites were administered in comparison to 150 parasites in our previous trial, which resulted in a higher liver fluke burden being observed (45-142; Fig. 5a). The aim of this analysis was to determine how liver fluke burden may impact parameters, such as eosinophilia, weight loss and anaemia, typically associated with fasciolosis (Fig. 5 and Fig. 6). The profile of eosinophilia was the same for both trials, with a predominance of these cells observed at 8 WPI (Fig. 5B), irrespective of the number of adult parasites recovered

406 at necropsy. In contrast, lower platelet counts were observed in the trial with higher
407 fluke burdens that were significantly different in the corresponding control and
408 vaccinated groups at 8 WPI and during the chronic phase of infection corresponding
409 to 20 WPI and 16 WPI depending on the trial schedule. The significantly different
410 platelet count observed between the two trials, indicates that sheep breed/age may
411 impact these cellular counts and should be considered when interpreting such results
412 (Fig. 5C).
413
414 As would be expected higher fluke burdens observed in the Cwiklinski et al. (2022)
415 trial data were associated with higher levels of circulating serum liver enzymes (Fig.
416 6). Analysis of the data using the GLDH PLT ratio scores indicated extensive damage
417 associated with *F. hepatica* infection occurred particularly at 8 WPI across all groups
418 independent of vaccine status (Fig. 7). This was also reflected in the corresponding
419 liver gross pathology scores at necropsy, of which 70 % of the livers had pathology
420 scores rated as moderate to severe. The higher GLDH PLT ratio values were also
421 associated with a trend towards lower haemoglobin levels compared with the current
422 trial, indicative of the potential for clinical signs associated with anaemia during the
423 later stages of infection with higher parasite burdens (Fig. 7B). Re-analysis of the data
424 in this context, reveals that there may be a threshold relating to the number of adult
425 parasites in terms of the utility of using the liver enzyme to PLT ratios to determine the
426 severity of liver pathology during the early phase of infection.
427
428 **4. Discussion**
429 We have embarked on a programme of discovery for vaccines against fasciolosis in
430 ruminants focused on candidates that are expressed and secreted by NEJ, the early

infective stage. Proteins whose functions have been shown to be involved in the parasite's interactions within the host environment were of prime interest as vaccine targets as our aim is to disrupt the critical processes involved in defence against/evasion of free radicals, and regulation of host and parasite proteases. Several of these vaccine antigens, such as FhPrx and FhTGR, have been trialled previously, with varying success, indicative of a common theme amongst *F. hepatica* vaccine trials of a lack of reproducible vaccine efficacy (reviewed by Spithill et al., 1999; Toet et al., 2014; Spithill et al., 2022). The results of the present vaccine study found that the three vaccine cocktails did not reduce the adult liver fluke burden, nor the egg number and viability. Limited effects on animal health parameters were also observed, with no differences in the weight gained over the course of the trial and no effect on the haemoglobin levels. In this study, modifications were made (addition of rFhSrp1 and reducing the stefin component to just rFhStf1) to the protease inhibitor vaccine cocktail that previously displayed detrimental effects on egg viability (Cwiklinski et al., 2022), which may have affected the outcome.

A factor contributing to the inconsistencies and variation observed for *F. hepatica* vaccine trials is adjuvant selection (Spithill et al., 2022). In our trials we aim to be consistent, using the same adjuvant, currently Montanide 61VG, which has been reported to be particularly suitable for enhancing the immunogenicity of antigens (Khorasani et al., 2016). This adjuvant was also used in the recent study combining rFhPrx with leucine aminopeptidase (rFhLAP), cathepsin L peptidase (rFhCL1) and helminth defence molecule (rFhHDM), which elicited significant vaccine protection in sheep against a challenge infection of 150 *F. hepatica* South Gloucester isolate metacercariae (reduction in adult fluke numbers of 37.2 %; Zafra et al., 2021). Other

456 vaccine studies, which include antioxidant molecules have used either Freund's
457 adjuvant/Freunds Incomplete adjuvant or Quil A, and have demonstrated significant
458 vaccine efficacy in rabbits (rFhTGR, Maggioli et al., 2011b) but not in cattle (rFhTGR;
459 Maggioli et al., 2016), or goats (rFhPrx; Mendes et al., 2010; Buffoni et al., 2012).
460 Freund's adjuvant is a powerful immunopotentiating formulation but is not suitable for
461 use in livestock (Stills, 2005) and thus identifying a safe alternative adjuvant is an
462 important criteria in early vaccine formulation studies and before downstream
463 commercialisation can be considered. However, discovery of the correct antigen
464 cocktail and adjuvant formulation presents a logistical and costly obstacle when using
465 ruminants in vaccine studies.
466
467 A consistent result observed in *F. hepatica* antioxidant vaccine studies including that
468 reported here is the increase of antigen-specific antibodies induced by vaccination.
469 The antibody profile observed in previous cattle and goat studies displayed an initial
470 increase following vaccination, which declined as the trial progressed (Mendes et al.,
471 2010; Buffoni et al., 2012; Maggioli et al., 2016), whereas the efficacious study in
472 rabbits displayed an initial delayed response around vaccination/parasite challenge
473 followed by a high antibody response that was maintained until the end of the trial
474 (Maggioli et al., 2011b). Similar high antibody levels were induced in rabbits
475 vaccinated with a recombinant fatty acid binding protein (rFh15), particularly when the
476 time between immunisation and challenge infection was increased (Casanueva et al.,
477 2001). In the present study a vaccination protocol was employed that differed to
478 previous studies and was inspired by the multiple doses of Barbervax given to protect
479 sheep against *Haemonchus contortus* (Broomfield et al., 2020); in this study two
480 vaccine administrations were given before challenge infection and three vaccine doses

after the challenge. This regime had the desired effect of maintaining the high antibody levels throughout the trial, albeit it did not induce significant protective effects. The general assumption is that higher antibody titres to vaccines would result in enhanced protection but the data suggests that selection of antigens and the immune response induced by adjuvant are more important factors to be considered. It should be noted that cellular immune responses were not assessed in this study.

It has been suggested that induction of IgG2 antibodies is correlated with protection against liver fluke in cattle (Mulcahy et al., 1998); however, the exact protective role of vaccine-induced antibodies against *F. hepatica* remains largely unexplored due to the lack of sufficient studies reporting vaccine efficacy. Elimination of the early stage NEJ parasites is thought to be mediated by antibody-dependent cell cytotoxicity (ADCC; reviewed by Spithill et al., 2022), therefore vaccines targeted against the early stage antigens are considered more promising as they would elicit antibodies that could induce ADCC killing of these stages. Antibodies likely also play a role in inactivating the proteins against which there are targeted, either by directly effecting protein activity or inducing opsonisation, by binding to the respective parasite native enzymes. However, as shown by Morrison et al. (1996), while vaccine-induced antibodies may have an effect on the activity of the respective vaccine antigen, this is not the sole mechanism involved in vaccine protection and reduced liver fluke burdens.

The selection of target molecules for the development of *F. hepatica* vaccines has been debated for the past three decades, with most studies opting for parasite molecules that are postulated to interact with the host, expected because of their presence in the excreted/secreted (ES) proteins and/or on the tegumental surface of

the parasites (Toet et al., 2014; Cwiklinski and Dalton, 2022; Spithill et al., 2022). Extensive biochemical analyses of the proteins used in this vaccine study has been carried out to confirm that our recombinant expression protocols produce correctly folded and functional proteins. With the exception of rFhTGR, all the proteins displayed functional activity in line with their predicted protein classification that infers that these molecules have the potential to act on host molecules (McGonigle et al., 1997; Sekiya et al., 2006; Smith et al., 2016; De Marco Verissimo et al., 2020; Calvani et al., 2022; Cwiklinski et al., 2022). It is also worth noting that while FhTGR has been observed in proteomic studies of the secreted proteins from NEJ (Lalor et al., 2021), it is not present with any great abundance and could explain the poor boosting of response following challenge infection.

Proteomic analysis has also revealed that in addition to being present within the ES protein fraction, all proteins in the vaccine cocktails are also contained within the extracellular vesicles (EV) derived from adult liver fluke ES (Murphy et al., 2020). Specific analysis of the EV composition from NEJ and immature fluke have yet to be carried out, however, given the transcript and protein profiles during these stages it is likely that the protease inhibitors and antioxidant molecules used are also present within NEJ EVs. Recent studies using parasite EV vaccines that contain a range of immunomodulatory proteins have shown protective immunity can be induced against subsequent infection (Trelis et al., 2016; Coakley et al., 2017; Shears et al., 2018; Chaiyadet et al., 2019; Mossallam et al., 2021). While targeting the immunomodulatory molecules contained within the *F. hepatica* EVs could be considered promising from a vaccine candidate selection point of view, the lack of protection by the vaccine cocktails in this study suggests these proteins may be hidden within the vesicles,

preventing their exposure to the host immune system during natural infections (Drurey and Maizels, 2021).

We have previously reported that vaccination against liver fluke can have a positive effect on animal health parameters, such as protecting against the weight loss typically observed with liver fluke infections (Cwiklinski et al., 2022). In this study, a marked difference was not observed between the weights of vaccinated and non-vaccinated animals. However, assessment of liver pathology markers based on human markers of fibrosis did present relevant insights into the influence of parasite burden on liver pathology and haematological parameters, indicating that severity of infection could be determined at 8 WPI when animals are infected with more than 45 parasites.

Comparative inter-trial analyses revealed haematological differences between the animals used in this study compared with previous trials. The two cohorts of animals used in these studies differed slightly in terms of sheep breed and age; 8-month-old Texel cross sheep were used by Cwiklinski et al. (2022) compared with the 4-month-old cross breed sheep used in this study. However, across the published reports of liver fluke vaccine trials in sheep there is large variability in trial design, with animals ranging from 4 months to 1 year old, representing a variety of different sheep breeds, including for example Merino (Perez-Caballero et al., 2018; Zafra et al., 2021), Corriedale (Maggioli et al., 2011a; Norbury et al., 2018; Wesolowska et al., 2018), Galician autochthonous breeds (Orbegozo-Medina et al., 2018) and Katahdin crossbreeds (Katahdin × East Friesian, Ortega-Vargas et al., 2019 Katahdin × Suffolk, Villa-Mancera et al., 2021). Recent studies have shown that sheep breed and age can impact vaccine efficacy and host responses to *Teladorsagia*

circumcincta infection (Gonzalez et al., 2019; Liu et al., 2022). The effects of these animal parameters have not been investigated for *F. hepatica* vaccine development; however, they may be contributing to the inconsistencies in vaccine efficacy observed between different trials.

Helminth vaccine trials are typically carried out under controlled experimental settings using oral infection of the parasite of interest. Field trials have been performed in cattle to assess the efficacy of a *Cooperia oncophora* vaccine, however only *Cooperia* egg counts and an assessment of *C. oncophora* and *Ostertagia ostertagi* larvae on pasture were performed in this study (Vlaminck et al., 2015). In this study, sheep were turned out onto pasture where they were exposed to a number of trichostrongyle gastrointestinal nematodes. Animals were monitored by FEC, which formed the basis on which drug treatment was administered according to standard practices (SCOPS b). This approach deviates from typical *F. hepatica* vaccine trials; however, it provides a more natural comparable setting to the typical parasite exposure that sheep are subjected to on pasture and is the scenario in which any suitable liver fluke vaccine must function. Moving forward, it will be important to consider the role of other pathogens and carry out vaccine trials in the field to assess the impact of co-infections on animal immune health and how they may influence our vaccine strategy particularly in relation to the type of adjuvant required to boost the most appropriate immune response.

Conclusion

To advance the development of liver fluke vaccines, we believe it is important to report data from trials whether they induce protection or not, particularly in regards to the

581 composition of the multivalent vaccines. Different combinations of antioxidants and
582 protease inhibitors, as well as other molecules that act at the host-parasite interface,
583 have shown some protective efficacy against *F. hepatica*, so there is a need to
584 continue to evaluate the liver fluke vaccine studies to discern which combinations of
585 proteins and adjuvants are likely to have the greatest effect. While parasite burden
586 and other parasitological measures such as egg counts have primarily been
587 considered the most important readout for vaccine efficacy, going forward a broader
588 approach is needed and, in particular, one that focuses more on parameters of animal
589 health and welfare.
590

References

Animal Health Ireland., 2021 Parasite Control – Liver Fluke, the facts. In Parasite Control Leaflet Series https://animalhealthireland.ie/assets/uploads/2021/06/PC-Liver-Fluke-2021.pdf?dl=1

Broomfield, M.A., Doyle, E.K., Kahn, L.P., Smith, W.D., Walkden-Brown, S.W., 2020. A simplified Barbervax(R) vaccination regimen in lambs to evoke immunological protection to *Haemonchus contortus*. Vet. Parasitol. 287, 109243. doi: 10.1016/j.vetpar.2020.109243.

Buffoni, L., Martinez-Moreno, F.J., Zafra, R., Mendes, R.E., Perez-Ecija, A., Sekiya, M., Mulcahy, G., Perez, J., Martinez-Moreno, A., 2012. Humoral immune response in goats immunised with cathepsin L1, peroxiredoxin and Sm14 antigen and experimentally challenged with *Fasciola hepatica*. Vet. Parasitol. 185, 315-321. doi: 10.1016/j.vetpar.2011.09.027.

Calvani, N.E.D., De Marco Verissimo, C., Jewhurst, H.L., Cwiklinski, K., Flaus, A., Dalton, J.P., 2022. Two distinct superoxidase dismutases (SOD) secreted by the helminth parasite *Fasciola hepatica* play roles in defence against metabolic and host immune cell-derived reactive oxygen species (ROS) during growth and development. Antioxidants (Basel) 11 (10), 1968. doi: 10.3390/antiox11101968.

Caminade, C., van Dijk, J., Baylis, M., Williams, D., 2015. Modelling recent and future climatic suitability for fasciolosis in Europe. Geospat. Health. 9, 301-308. doi: 10.4081/gh.2015.352.

Casanueva, R., Hillyer, G.V., Ramajo, V., Oleaga, A., Espinoza, E.Y., Muro, A., 2001. Immunoprophylaxis against *Fasciola hepatica* in rabbits using a recombinant Fh15 fatty acid-binding protein. J. Parasitol. 87, 697-700. doi: 10.1645/0022-3395(2001)087[0697:IAFHIR]2.0.CO;2.

Chaiyadet, S., Sotillo, J., Krueajampa, W., Thongsen, S., Brindley, P.J., Sripa, B., Loukas, A., Laha, T., 2019. Vaccination of hamsters with *Opisthorchis viverrini* extracellular vesicles and vesicle-derived recombinant tetraspanins induces antibodies that block vesicle uptake by cholangiocytes and reduce parasite burden after challenge infection. PLoS Negl. Trop. Dis. 13, e0007450. doi: 10.1371/journal.pntd.0007450.

Charlier, J., Rinaldi, L., Musella, V., Ploeger, H.W., Chartier, C., Vineer, H.R., Hinney, B., von Samson-Himmelstjerna, G., Băcescu, B., Mickiewicz, M., Mateus, T.L., Martinez-Valladares, M., Quealy, S., Azaizeh, H., Sekovska, B., Akkari, H., Petkevicius, S., Hektoen, L., Höglund, J., Morgan, E.R., Bartley, D.J., Claerebout, E., 2020. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. Prev. Vet. Med. 182, 105103. doi: 10.1016/j.prevetmed.2020.105103.

Coakley, G., McCaskill, J.L., Borger, J.G., Simbari, F., Robertson, E., Millar, M., Harcus, Y., McSorley, H.J., Maizels, R.M., Buck, A.H., 2017. Extracellular vesicles from a helminth parasite suppress macrophage activation and constitute an effective vaccine for protective immunity. Cell Rep. 19, 1545-1557. doi: 10.1016/j.celrep.2017.05.001.

Collins, P.R., Stack, C.M., O'Neill, S.M., Doyle, S., Ryan, T., Brennan, G.P., Mousley, A., Stewart, M., Maule, A.G., Dalton, J.P., Donnelly, S., 2004. Cathepsin L1, the major protease involved in liver fluke (*Fasciola hepatica*) virulence: propetide cleavage sites and autoactivation of the zymogen secreted from gastrodermal cells. J. Biol. Chem. 279, 17038-17046. doi: 10.1074/jbc.M308831200.

COWS, Control of Worms Sustainably. Liver and Rumen Fluke. https://www.cattleparasites.org.uk/liver-fluke-and-rumen-fluke/

Cwiklinski, K., Dalton, J.P., 2022. Omics tools enabling vaccine discovery against fasciolosis. Trends Parasitol. 38, 1068-1079. doi: 10.1016/j.pt.2022.09.009.

Cwiklinski, K., Dalton, J.P., Dufresne, P.J., La Course, J., Williams, D.J., Hodgkinson, J., Paterson, S., 2015. The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biol. 16 (1), 71. doi: 10.1186/s13059-015-0632-2.

Cwiklinski, K., Drysdale, O., López Corrales, J., Corripio-Miyar, Y., De Marco Verissimo, C., Jewhurst, H., Smith, D., Lalor, R., McNeilly, T.N., Dalton, J.P., 2022. Targeting secreted protease/anti-

protease balance as a vaccine strategy against the helminth *Fasciola hepatica*. Vaccines (Basel) 10 (2), 155. doi: 10.3390/vaccines10020155.

De Marco Verissimo, C., Jewhurst, H.L., Dobó, J., Gál, P., Dalton, J.P., Cwiklinski, K., 2022. *Fasciola hepatica* is refractory to complement killing by preventing attachment of mannose binding lectin (MBL) and inhibiting MBL-associated serine proteases (MASPs) with serpins. PLoS Pathog. 18, e1010226. doi: 10.1371/journal.ppat.1010226.

De Marco Verissimo, C., Jewhurst, H.L., Tikhonova, I.G., Urbanus, R.T., Maule, A.G., Dalton, J.P., Cwiklinski, K., 2020. *Fasciola hepatica* serine protease inhibitor family (serpins): Purposely crafted for regulating host proteases. PLoS Negl. Trop. Dis. 14, e0008510. doi: 10.1371/journal.pntd.0008510.

Dorey, A., Cwiklinski, K., Rooney, J., De Marco Verissimo, C., López Corrales, J., Jewhurst, H., Fazekas, B., Calvani, N.E.D., Hamon, S., Gaughan, S., Dalton, J.P., Lalor, R., 2021. Autonomous non antioxidant roles for *Fasciola hepatica* secreted Thioredoxin-1 and Peroxiredoxin-1. Front. Cell. Infect. Microbio.l 11, 667272. doi: 10.3389/fcimb.2021.667272.

Drurey, C., Maizels, R.M., 2021. Helminth extracellular vesicles: Interactions with the host immune system. Mol. Immunol. 137, 124-133. doi: 10.1016/j.molimm.2021.06.017.

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

Gonzalez, J.F., Hernandez, J.N., Machin, C., Perez-Hernandez, T., Wright, H.W., Corripio-Miyar, Y., Price, D.R.G., Matthews, J.B., McNeilly, T.N., Nisbet, A.J., 2019. Impacts of breed type and vaccination on *Teladorsagia circumcincta* infection in native sheep in Gran Canaria. Vet. Res. 50, 29. doi: 10.1186/s13567-019-0646-y.

Hume, D.A., Whitelaw, C.B.A., Archibald, A.L., 2011. The future of animal production: improving productivity and sustainability. J. Agric. Sci. 149, 9-16. doi: 10.1017/S0021859610001188

Jackson, P.G.G., Cockcroft, P.D. (Eds), 2002. Clinical examination of farm animals. Appendix 3 Laboratory references values: biochemistry. Blackwell Science, UK, 303-305 pp.

Khorasani, A., Madadgar, O., Soleimanjahi, H., Keyvanfar, H., Mahravani, H., 2016. Evaluation of the efficacy of a new oil-based adjuvant ISA 61 VG FMD vaccine as a potential vaccine for cattle. Iran. J. Vet. Res. 17, 8-12.

Lalor, R., Cwiklinski, K., Calvani, N.E.D., Dorey, A., Hamon, S., Corrales, J.L., Dalton, J.P., De Marco Verissimo, C., 2021. Pathogenicity and virulence of the liver flukes *Fasciola hepatica* and *Fasciola gigantica* that cause the zoonosis Fasciolosis. Virulence. 12, 2839-2867. doi: 10.1080/21505594.2021.1996520.

Liu, W., McNeilly, T.N., Mitchell, M., Burgess, S.T.G., Nisbet, A.J., Matthews, J.B., Babayan, S.A., 2022. Vaccine-induced time- and age-dependent mucosal immunity to gastrointestinal parasite infection. NPJ Vaccines. 7, 78. doi: 10.1038/s41541-022-00501-0.

López Corrales, J., Cwiklinski, K., De Marco Verissimo, C., Dorey, A., Lalor, R., Jewhurst, H., McEvoy, A., Diskin, M., Duffy, C., Cosby, S.L., Keane, O.M., Dalton, J.P., 2021. Diagnosis of sheep fasciolosis caused by *Fasciola hepatica* using cathepsin L enzyme-linked immunosorbent assays (ELISA). Vet. Parasitol. 298, 109517. doi: 10.1016/j.vetpar.2021.109517.

MacGillivray, F., de Waal, T., D, M., Taylor, M., V, B., R, D., T, J., B, R., Forbes, A., 2013. An abattoir survey to determine the population profile in the autumn of *Fasciola hepatica* in condemned bovine livers from Ireland and the United Kingdom. J. Appl. Res. Vet. Med. 11, 1-6.

Maggioli, G., Acosta, D., Silveira, F., Rossi, S., Giacaman, S., Basika, T., Gayo, V., Rosadilla, D., Roche, L., Tort, J., Carmona, C., 2011a. The recombinant gut-associated M17 leucine aminopeptidase in combination with different adjuvants confers a high level of protection against *Fasciola hepatica* infection in sheep. Vaccine. 29, 9057-9063. doi: 10.1016/j.vaccine.2011.09.020.

690 Maggioli, G., Bottini, G., Basika, T., Alonzo, P., Salinas, G., Carmona, C., 2016. Immunization with
691 *Fasciola hepatica* thioredoxin glutathione reductase failed to confer protection against
692 fasciolosis in cattle. Vet. Parasitol. 224, 13-19. doi: 10.1016/j.vetpar.2016.05.007.
693 Maggioli, G., Silveira, F., Martin-Alonso, J.M., Salinas, G., Carmona, C., Parra, F., 2011b. A recombinant
694 thioredoxin-glutathione reductase from *Fasciola hepatica* induces a protective response in
695 rabbits. Exp. Parasitol. 129, 323-330. doi: 10.1016/j.exppara.2011.09.013.
696 Mazeri, S., Rydevik, G., Handel, I., Bronsvoort, B.M.D., Sargison, N., 2017. Estimation of the impact of
697 *Fasciola hepatica* infection on time taken for UK beef cattle to reach slaughter weight. Sci.
698 Rep. 7, 7319. doi: 10.1038/s41598-017-07396-1.
699 McGonigle, S., Curley, G.P., Dalton, J.P., 1997. Cloning of peroxiredoxin, a novel antioxidant enzyme,
700 from the helminth parasite *Fasciola hepatica*. Parasitol. 115, 101-104. doi:
701 10.1017/s0031182097001170.
702 Mendes, R.E., Perez-Ecija, R.A., Zafra, R., Buffoni, L., Martinez-Moreno, A., Dalton, J.P., Mulcahy, G.,
703 Perez, J., 2010. Evaluation of hepatic changes and local and systemic immune responses in
704 goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola*
705 *hepatica*. Vaccine. 28, 2832-2840. doi: 10.1016/j.vaccine.2010.01.055.
706 Morgan, E.R., Charlier, J., Hendrickx, G., Biggeri, A., Catalan, D., von Samson-Himmelstjerna, G.,
707 Demeler, J., Müller, E., van Dijk, J., Kenyon, F., Skuce, P., Höglund, J., O'Kiely, P., van Ranst, B.,
708 de Waal, T., Rinaldi, L., Cringoli, G., Hertzberg, H., Torgerson, P., Wolstenholme, A.,
709 Vercruysse, J., 2013. Global Change and Helminth Infections in Grazing Ruminants in Europe:
710 Impacts, Trends and Sustainable Solutions. Agriculture. 3, 484-502. doi:
711 10.3390/agriculture3030484.
712 Morrison, C.A., Colin, T., Sexton, J.L., Bowen, F., Wicker, J., Friedel, T., Spithill, T.W., 1996. Protection
713 of cattle against *Fasciola hepatica* infection by vaccination with glutathione S-transferase.
714 Vaccine. 14(17-18):1603-12. doi: 10.1016/s0264-410x(96)00147-8.
715 Mossallam, S.F., Abou-El-Naga, I.F., Abdel Bary, A., Elmorsy, E.A., Diab, R.G., 2021. *Schistosoma*
716 *mansoni* egg-derived extracellular vesicles: A promising vaccine candidate against murine
717 schistosomiasis. PLoS Negl. Trop. Dis. 15, e0009866. doi: 10.1371/journal.pntd.0009866.
718 Mulcahy, G., O'Connor, F., McGonigle, S., Dowd, A., Clery, D.G., Andrews, S.J., Dalton, J.P., 1998
719 Correlation of specific antibody titre and avidity with protection in cattle immunized against
720 *Fasciola hepatica*. Vaccine. 16(9-10):932-9. doi: 10.1016/s0264-410x(97)00289-2.
721 Murphy, A., Cwiklinski, K., Lalor, R., O'Connell, B., Robinson, M.W., Gerlach, J., Joshi, L., Kilcoyne, M.,
722 Dalton, J.P., O'Neill, S.M., 2020. *Fasciola hepatica* Extracellular Vesicles isolated from
723 excretory-secretory products using a gravity flow method modulate dendritic cell phenotype
724 and activity. PLoS Negl. Trop. Dis. 14, e0008626. doi: 10.1371/journal.pntd.0008626.
725 Norbury, L.J., Basalaj, K., Zawistowska-Deniziak, A., Sielicka, A., Wilkowski, P., Wesolowska, A.,
726 Smooker, P.M., Wedrychowicz, H., 2018. Intranasal delivery of a formulation containing stage-
727 specific recombinant proteins of *Fasciola hepatica* cathepsin L5 and cathepsin B2 triggers an
728 anti-fecundity effect and an adjuvant-mediated reduction in fluke burden in sheep. Vet.
729 Parasitol. 258, 14-23. doi: 10.1016/j.vetpar.2018.05.008.
730 Nyirenda, S.S., Sakala, M., Moonde, L., Kayesa, E., Fandamu, P., Banda, F., Sinkala, Y., 2019. Prevalence
731 of bovine fascioliasis and economic impact associated with liver condemnation in abattoirs in
732 Mongu district of Zambia. BMC Vet. Res. 15, 33. doi: 10.1186/s12917-019-1777-0.
733 Orbegozo-Medina, R.A., Martinez-Sernandez, V., Gonzalez-Warleta, M., Castro-Hermida, J.A., Mezo,
734 M., Ubeira, F.M., 2018. Vaccination of sheep with Quil-A(R) adjuvant expands the antibody
735 repertoire to the *Fasciola* MF6p/FhHDM-1 antigen and administered together impair the
736 growth and antigen release of flukes. Vaccine. 36, 1949-1957. doi:
737 10.1016/j.vaccine.2018.02.115.
738 Ortega-Vargas, S., Espitia, C., Sahagún-Ruiz, A., Parada, C., Balderas-Loaeza, A., Villa-Mancera, A.,
739 Quiroz-Romero, H., 2019. Moderate protection is induced by a chimeric protein composed of

leucine aminopeptidase and cathepsin L1 against *Fasciola hepatica* challenge in sheep. Vaccine. 37, 3234-3240. doi: 10.1016/j.vaccine.2019.04.067.

Perez-Caballero, R., Siles-Lucas, M., Gonzalez-Miguel, J., Martinez-Moreno, F.J., Escamilla, A., Perez, J., Martinez-Moreno, A., Buffoni, L., 2018. Pathological, immunological and parasitological study of sheep vaccinated with the recombinant protein 14-3-3z and experimentally infected with *Fasciola hepatica*. Vet. Immunol. Immunopathol. 202, 115-121. doi: 10.1016/j.vetimm.2018.07.006.

Sangster, N.C., Martinez-Moreno, A., Perez, J., 2022. Pathology, pathophysiology and clinical aspects, In: Dalton, J.P. (Ed.) Fasciolosis. CAB International, Wallingford, Oxfordshire, UK, pp. 145-179.

SCOPS a, Sustainable Control of Parasites in Sheep. Liver Fluke https://www.scops.org.uk/internal-parasites/liver-fluke/

SCOPS b, Sustainable Control of Parasites in Sheep. Worms – Choosing a product https://www.scops.org.uk/internal-parasites/worms/choosing-a-product/

Sekiya, M., Mulcahy, G., Irwin, J.A., Stack, C.M., Donnelly, S.M., Xu, W., Collins, P., Dalton, J.P., 2006. Biochemical characterisation of the recombinant peroxiredoxin (FhePrx) of the liver fluke, *Fasciola hepatica*. FEBS Lett. 580, 5016-5022. doi: 10.1016/j.febslet.2006.08.019.

Shears, R.K., Bancroft, A.J., Hughes, G.W., Grencis, R.K., Thornton, D.J., 2018. Extracellular vesicles induce protective immunity against *Trichuris muris*. Parasite. Immunol. 40, e12536. doi: 10.1111/pim.12536.

Smith, D., Cwiklinski, K., Jewhurst, H., Tikhonova, I.G., Dalton, J.P., 2020. An atypical and functionally diverse family of Kunitz-type cysteine/serine proteinase inhibitors secreted by the helminth parasite *Fasciola hepatica*. Sci. Rep. 10, 20657. doi: 10.1038/s41598-020-77687-7.

Smith, D., Tikhonova, I.G., Jewhurst, H.L., Drysdale, O.C., Dvořák, J., Robinson, M.W., Cwiklinski, K., Dalton, J.P., 2016. Unexpected Activity of a Novel Kunitz-type Inhibitor: inhibition of cysteine proteases but not serine proteases. J. Biol. Chem. 291, 19220-19234. doi: 10.1074/jbc.M116.724344.

Spithill, T., Toet, H., Rathinasamy, V., Zerna, G., Swan, J., Cameron, T.C., Smooker, P.M., Piedrafita, D.M., Dempster, R., Beddoe, T., 2022. Vaccines for *Fasciola*: New thinking for an old problem, In: Dalton, J.P. (Ed.) Fasciolosis. CAB International, Wallingford, Oxfordshire, UK, pp. 379-422.

Spithill, T.W., Smooker, P.M., Sexton, J.L., Bozas, E., Morrison, C.A., Creaney, J., Parsons, J.C., 1999. Development of vaccines against *Fasciola hepatica*, In: Dalton, J.P. (Ed.) Fasciolosis. CABI Publishing, pp. 377-410.

Stills, H.F., Jr., 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J. 46, 280-293. doi: 10.1093/ilar.46.3.280.

Stuen, S., Ersdal, C. 2022. Fasciolosis - An increasing challenge in the sheep industry. Animals (Basel). 12, 1491. doi: 10.3390/ani12121491.

Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. Int. J. Parasitol. 44, 915-27. doi: 10.1016/j.ijpara.2014.07.011.

Trelis, M., Galiano, A., Bolado, A., Toledo, R., Marcilla, A., Bernal, D., 2016. Subcutaneous injection of exosomes reduces symptom severity and mortality induced by *Echinostoma caproni* infection in BALB/c mice. Int. J. Parasitol. 46, 799-808. doi: 10.1016/j.ijpara.2016.07.003.

University of Guelph, Biochemistry reference intervals. https://www.uoguelph.ca/ahl/biochemistry-reference-intervals.

Utrera-Quintana, F., Covarrubias-Balderas, A., Olmedo-Juarez, A., Cruz-Avina, J., Cordova-Izquierdo, A., Perez-Mendoza, N., Villa-Mancera, A., 2022. Fasciolosis prevalence, risk factors and economic losses due to bovine liver condemnation in abattoirs in Mexico. Microb. Pathog. 173, 105851. doi: 10.1016/j.micpath.2022.105851.

Villa-Mancera, A., Olivares-Perez, J., Olmedo-Juarez, A., Reynoso-Palomar, A., 2021. Phage display-based vaccine with cathepsin L and excretory-secretory products mimotopes of *Fasciola hepatica* induces protective cellular and humoral immune responses in sheep. Vet. Parasitol. 289, 109340. doi: 10.1016/j.vetpar.2020.109340.

Vlaminck, J., Borloo, J., Vercruysse, J., Geldhof, P., Claerebout, E., 2015. Vaccination of calves against *Cooperia oncophora* with a double-domain activation-associated secreted protein reduces parasite egg output and pasture contamination. Int J Parasitol. 45(4):209-13. doi: 10.1016/j.ijpara.2014.11.001.

Wai, C.T., Greenson, J.K., Fontana, R.J., Kalbfleisch, J.D., Marrero, J.A., Conjeevaram, H.S., Lok, A.S., 2003. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 38, 518-526. doi: 10.1053/jhep.2003.50346.

Wesołowska, A., Kozak Ljunggren, M., Jedlina, L., Basałaj, K., Legocki, A., Wędrychowicz, H., Kesik-Brodacka, M., 2018. A preliminary study of a lettuce-based edible vaccine expressing the cysteine proteinase of *Fasciola hepatica* for fasciolosis control in livestock. Front. Immunol. 9, 2592. doi: 10.3389/fimmu.2018.02592.

Zafra, R., Buffoni, L., Pérez-Caballero, R., Molina-Hernández, V., Ruiz-Campillo, M.T., Pérez, J., Martínez-Moreno, Á., Martínez Moreno, F.J., 2021. Efficacy of a multivalent vaccine against *Fasciola hepatica* infection in sheep. Vet. Res. 52, 13. doi: 10.1186/s13567-021-00895-0.

CRediT authorship contribution statement

Krystyna Cwiklinski: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Amanda McEvoy**: Formal analysis, Investigation, Data Curation, Writing – review & editing, Project administration. **Jesús López Corrales**: Validation, Formal analysis, Investigation, Data Curation, Writing – review & editing, Visualization. **Heather Jewhurst**: Investigation, Writing – review & editing. **Nichola Eliza Davies Calvani**: Investigation, Writing – review & editing. **Carolina De Marco Verissimo**: Investigation, Writing – review & editing. **Amber Louise Dorey**: Investigation, Writing – review & editing. **Orla M. Keane**: Conceptualization, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration. **John Pius Dalton**: Conceptualization, Methodology, Validation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Richard Lalor**: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by Science Foundation Ireland (SFI) Professorship grant (17/RP/5368) awarded to J.P. Dalton.

Figure Legends

Fig. 1. Purification of recombinant proteins. 4-20 % SDS-PAGE analysis of the recombinant vaccine antigens. (A) G1: Antioxidants *Fasciola hepatica* peroxiredoxin (rFhPrx), thioredoxin (rFhTrx) and thioredoxin-glutathione reductase (rFhTGR), expressed in an *E. coli* expression system. (B) G2: Antioxidants *F. hepatica* peroxiredoxin (rFhPrx), thioredoxin (rFhTrx), and two superoxide dismutase proteins (rFhSOD1 and rFhSOD3), expressed in an *E. coli* expression system. (C) G3: Inhibitors *F. hepatica* Kunitz-type inhibitor (rFhKT1) and stefin (rFhStf1) expressed in a yeast expression system and serpin (rFhSrp1) expressed in *E. coli*.

Fig.2. Total IgG responses to the vaccine antigens. Mean optical density (OD) values for total IgG antibodies to the vaccine antigens in the three vaccine groups (A: G1; B: G2; C: G3) and infected control group (D: G4) at six time-points during the vaccine trial. Antibody responses to *Fasciola hepatica* infection are displayed as mean OD values for total IgG antibodies to the recombinant cathepsin L peptidase in all four groups. Data represented as ± standard deviation. Arrows highlighting when the

847 animals were vaccinated (Vac) and infected with *F. hepatica* metacercariae (Inf) are
848 shown on each graph.
849
850 **Fig.3. Measures of animal health affected by fasciolosis.** (A) Graphical
851 representation of total weight gain ± standard deviation during the vaccine trial,
852 calculated as the difference between the weight at week 23 and the starting weight at
853 the beginning of the trial. (B) Levels of haemoglobin (± standard deviation) measured
854 as g/dL in blood samples taken at 8 weeks post infection (WPI) and 20 WPI. Normal
855 values typically range between 10 and 15 g/dL as shown by the dashed lines.
856
857 **Fig.4. Associations between markers of fasciolosis.** (A) Comparison between
858 adult fluke number at necropsy with the ratio of the level of glutamate dehydrogenase
859 (GLDH; units/l) in serum compared with the number of platelets in blood samples
860 (PLT) at 8 weeks post infection (WPI) (GLDH PLT ratio). (B) Comparison between
861 level of haemoglobin at 8 WPI (g/dL) with a ratio of the level of glutamate
862 dehydrogenase (GLDH; units/l) in serum compared with the number of platelets in
863 blood samples (PLT) at 8 WPI. G1, vaccine group 1 (blue); G2, vaccine group 2 (red);
864 G3, vaccine group 3 (black); G4, control *Fasciola hepatica* infected group (purple).
865 Dashed line represents a ratio of GLDH PLT of 0.5, below which fibrosis is not
866 indicated.
867
868 **Fig.5. Comparison of *Fasciola hepatica* vaccine trials: Profile of haematology**
869 **parameters.** Markers of *F. hepatica* infection from this vaccine trial were compared
870 with the previously published study by Cwiklinski et al. (2022) carried out at the
871 Moredun Research Institute, evaluating a *F. hepatica* protease inhibitor vaccine

cocktail. T_G3: group 3, animals vaccinated with the *F. hepatica* inhibitor cocktail in Teagasc trial, grey. T_G4: group 4, control infected group in Teagasc trial, black. M_G2: group 2, control infected group in Moredun trial, dark blue. M_G3 and M_G4: group 3 (teal) and group 4 (light blue), respectively, representing animals vaccinated with the *F. hepatica* inhibitor cocktail in Moredun trial. (A) Graphical representation of the number of adult flukes recovered at necropsy at the end of the respective trials. (B) Level of eosinophilia during the vaccine trial time-course represented as a percentage of the total cells, calculated from blood samples taken at three time-points, 0 weeks post infection (WPI), 8 WPI and 20 WPI (Teagasc trial) or 16 WPI (Moredun trial) ± standard deviation. (C) Number of platelets (PLT; x 10^9/L) in blood samples taken at 0 WPI, 8 WPI and 20 WPI (Teagasc trial) or 16 WPI (Moredun trial) ± standard deviation. Statistical analyses were carried out by separately comparing the control infected groups and the vaccine groups in each trial (c and h: $P < 0.05$; f: $P < 0.01$; a, b, d, e and g: $P < 0.001$).

Fig.6. Comparison of *Fasciola hepatica* vaccine trials: Profile of liver enzymes. Markers of *F. hepatica* infection from this vaccine trial were compared with the previously published study by Cwiklinski et al. (2022) carried out at the Moredun Research Institute, evaluating the *F. hepatica* inhibitor vaccine cocktail. T_G3: group 3, animals vaccinated with the *F. hepatica* inhibitor cocktail in Teagasc trial, grey. T_G4: group 4, control infected group in Teagasc trial, black. M_G2: group 2, control infected group in Moredun trial, dark blue. M_G3 and M_G4: group 3 (teal) and group 4 (light blue), respectively, representing animals vaccinated with the *F. hepatica* inhibitor cocktail in Moredun trial. (A) Level of glutamate dehydrogenase (GLDH; units/L) in serum analysed at three time-points, 0 weeks post infection (WPI), 8 WPI

and 20 WPI (Teagasc trial) or 16 WPI (Moredun trial), ± standard deviation. (B) Level of gamma glutamyl-transferase (GGT; units/l) in serum analysed at three time-points, 0 WPI, 8 WPI and 20 WPI (Teagasc trial) or 16 WPI (Moredun trial), ± standard deviation. Statistical analyses were carried out by separately comparing the control infected groups and the vaccine groups in each trial (a: P <0.01; b: P <0.001).

Fig.7. Comparison of *Fasciola hepatica* vaccine trials: GLDH PLT ratio at 8 weeks post infection (WPI). Ratio of glutamate dehydrogenase (GLDH; units/l) in serum to the number of platelets in blood samples (PLT) at 8 WPI was compared to (A) the number of adult flukes recovered at necropsy and (B) the level of haemoglobin at 8 WPI (g/dL), using data from this study and the study previously published by Cwiklinski et al. (2022). This study: T_G3: vaccine group 3, black; T_G4: control *F. hepatica* infected group, purple. Cwiklinski et al. (2022) study: M_G2: control *F. hepatica* infected group, grey; M_G3: vaccine group 1, orange; M_G4: vaccine group 2, gold. Dashed line represents a ratio of GLDH PLT of 0.5, below which fibrosis is not indicated.

Supplementary Material

Supplementary Table. S1. Profile of nematode infection based on eggs in faeces following exposure to pasture.

Supplementary Fig. S1. Profile of weight gain over the 24 weeks of the trial displayed as daily weight gain (DWG). Graphical representation of the DWG calculated at four-week intervals ± standard deviation for the four animal groups. (A)

G1, vaccine group 1 (blue); (B) G2, vaccine group 2 (red); (C) G3, vaccine group 3 (black); (D) G4, control *F. hepatica* infected group (purple).

Supplementary Fig.S2. Comparison between adult fluke number at necropsy with a ratio of the level of liver enzymes in serum compared with the number of platelets in blood samples (PLT). (A-C) Ratio derived from the level of glutamate dehydrogenase (GLDH; units/l) in serum compared with the number of platelets in blood samples (PLT) at 8 weeks post infection (WPI), 20 WPI and 26 WPI, respectively. (D-F) Ratio derived from the level of gamma glutamyl-transferase (GGT; units/l) in serum compared with the number of platelets in blood samples (PLT) at 8 WPI, 20 WPI and 26 WPI, respectively. G1, vaccine group 1 (blue); G2, vaccine group 2 (red); G3, vaccine group 3 (black); G4, control *Fasciola hepatica* infected group (purple). Dashed line represents a ratio of liver enzyme PLT of 0.5, below which fibrosis is not indicated.

Supplementary Fig.S3. Comparison between level of haemoglobin with a ratio of the level of liver enzymes in serum compared with the number of platelets in blood samples (PLT). (A-C) Ratio derived from the level of glutamate dehydrogenase (GLDH; units/l) in serum compared with the number of platelets in blood samples (PLT) at 8 weeks post infection (WPI), 20 WPI and 26 WPI, respectively. (D-F) Ratio derived from the level of gamma glutamyl-transferase (GGT; units/l) in serum compared with the number of platelets in blood samples (PLT) at 8 WPI, 20 WPI and 26 WPI, respectively. G1, vaccine group 1 (blue); G2, vaccine group 2 (red); G3, vaccine group 3 (black); G4, control *Fasciola hepatica* infected group (purple). Dashed line represents a ratio of liver enzyme PLT of 0.5, below which fibrosis is not indicated.

Supplementary Fig.S4. Graphical representation of liver pathology scores. (A) Scoring system 0: No signs of damage/infection; 1: Mild local fibrosis/damage; 2: Severe local fibrosis/damage or mild generalised fibrosis/damage; 3; Severe local fibrosis/damage and calcified bile ducts or severe generalised fibrosis/damage. (B) Correlation analysis between liver pathology score and number of adult flukes recovered at post-mortem (Spearman correlation, $r = 0.4370$; $p < 0.001$). G1, vaccine group 1 (blue); G2, vaccine group 2 (red); G3, vaccine group 3 (black); G4, control *Fasciola hepatica* infected group (purple).

Table 1. Expected molecular sizes of the recombinantly expressed *Fasciola hepatica* proteins.

Protein	Gene id	Molecular size (kDa)	Reference
Peroxiredoxin (FhPrx)	U88577 maker-scaffold10x_439_pilon-augustus-gene-0.21	25	Sekiya et al., 2006; this study
Thioredoxin (FhTrx)	AJ250097 maker-scaffold10x_143_pilon-augustus-gene-0.62	12	Dorey et al., 2021; this study
Thioredoxin-glutathione reductase (FhTGR)	AM709787 maker-scaffold10x_303_pilon-snap-gene-0.30	66	Maggioli et al., 2011
Superoxide dismutase 1 (FhSOD1)	AF071229 BN1106_s3189B000243 maker-scaffold10x_61_pilon-snap-gene-0.36	16	Calvani et al., 2022
Superoxide dismutase 3 (FhSOD3)	BN1106_s4478B000037 maker-scaffold10x_713_pilon-snap-gene-0.105	18	Calvani et al., 2022
Kunitz-type inhibitor (FhKT1)	BN1106_s8826B000029	6	Smith et al., 2016
Stefin 1 (FhStf1)	BN1106_s4651B000094 maker-scaffold10x_815_pilon-snap-gene-1.92	11	Cwiklinski et al., 2022

Serpin 1 (FhSrp1)	MT419773 BN1106_s3864B000104 maker-scaffold10x_114_pilon-snap-gene-0.90	41	De Marco Verissimo et al., 2020

958

959

960

961 Table 2. Number of adult liver fluke recovered at necropsy.

Group^	Mean fluke burden ±SD (Adult flukes recovered per animal)	Range	% protection
G1	37.47 ± 14.95 (16,22,22,23,26,29,32,40,43,43,44,46,47,62,67)	16-67	0
G2	35.93 ± 14.61 (4,23,23,25,26,29,34,35,41,43,45,49,51,51,60)	4-60	0
G3	32.47 ± 8.643 (20,22,23,23,26,29,30,31,35,36,39,41,42,45,45)	20-45	0
G4	31.53 ± 13.33 (8,17,18,19,21,27,32,32,33,36,41,41,42,50,56)	8-56	

962 ^G1: vaccinated with vaccine 1 (rFhPrx, rFhTrx, rFhTGR); G2: vaccinated with vaccine 2
963 (rFhPrx, rFhSOD1, rFhSOD3, rFhTrx); G3: vaccinated with vaccine 3 (rFhKT1, rFhSrp1,
964 rFhStf1); G4: control, unvaccinated and infected.

965

966

967 Table 3. Faecal egg count represented as egg per gram (EPG).

	Mean FEC (EPG) ± SD		
Group^	18 WPI	22 WPI	26 WPI
G1	220.2 ± 94.29	278.5 ± 166.4	364.6 ± 197.9
G2	242.1 ± 109.0	265.8 ± 91.73	331.6 ± 140.5
G3	229.3 ± 67.52	257.7 ± 116.7	305.3 ± 104.3
G4	195.4 ± 90.29	199.2 ± 85.95	253.2 ± 131.0

968 WPI, weeks post-infection

969 ^G1: vaccinated with vaccine 1 (rFhPrx, rFhTrx, rFhTGR); G2: vaccinated with vaccine 2
970 (rFhPrx, rFhSOD1, rFhSOD3, rFhTrx); G3: vaccinated with vaccine 3 (rFhKT1, rFhSrp1,
971 rFhStf1); G4: control, unvaccinated and infected.

972

973 Table 4. Eggs recovered from gall bladder at necropsy and relative egg embryonation and
974 hatch rate.

Group^	Number of eggs ± SD	% Embryonation	% Hatch rate	% protection *
G1	66,233 ± 84,483	85.3 ± 13.2	58.6 ± 17.1	0
G2	173,287 ± 271,268	79.1 ± 15.5	50.0 ± 15.6	8.7
G3	81,693 ± 75,249	85.5 ± 9.0	58.6 ± 12.4	0
G4	49,370 ± 62,360	84.4 ± 7.2	54.8 ± 14.6	

975 ^G1: vaccinated with vaccine 1 (rFhPrx, rFhTrx, rFhTGR); G2: vaccinated with vaccine 2
976 (rFhPrx, rFhSOD1, rFhSOD3, rFhTrx); G3: vaccinated with vaccine 3 (rFhKT1, rFhSrp1,
977 rFhStf1); G4: control, unvaccinated and infected.

978 *Based on mean hatch rate relative to the egg hatch rate of eggs recovered from G4 control
979 infected animals.

980

Figure 1

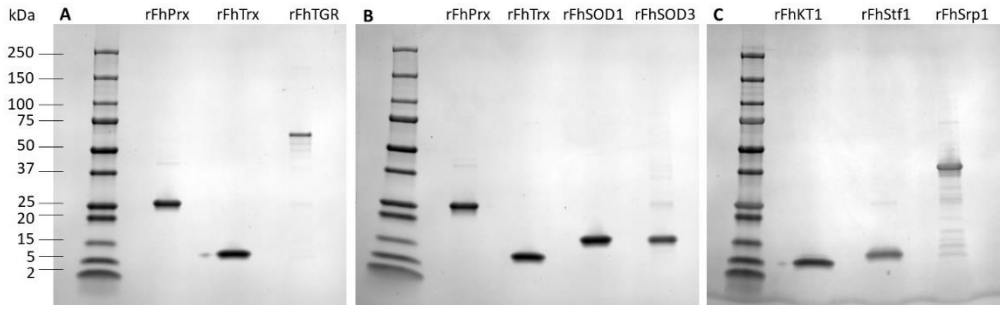


Figure 2

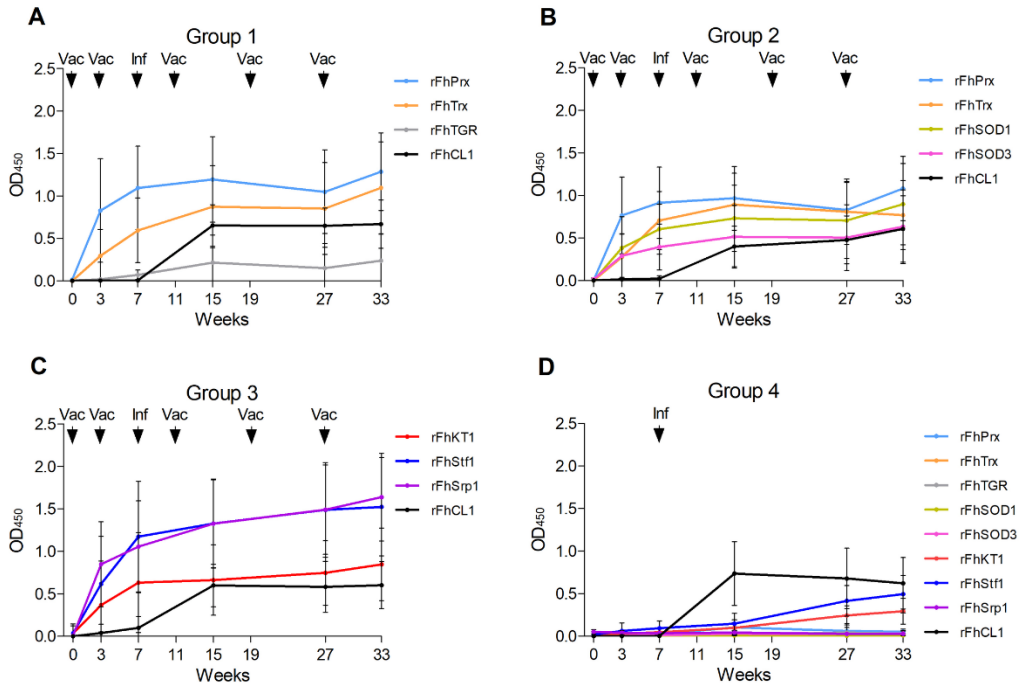
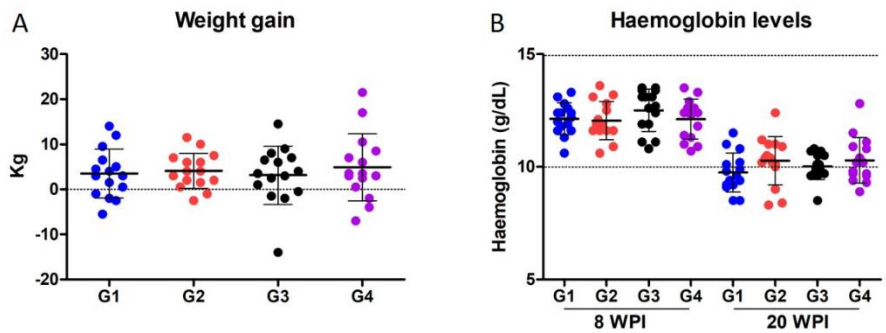
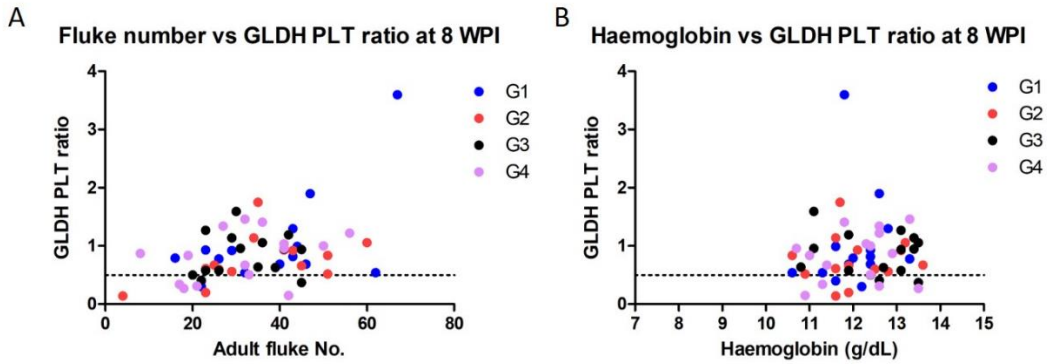


Figure 3



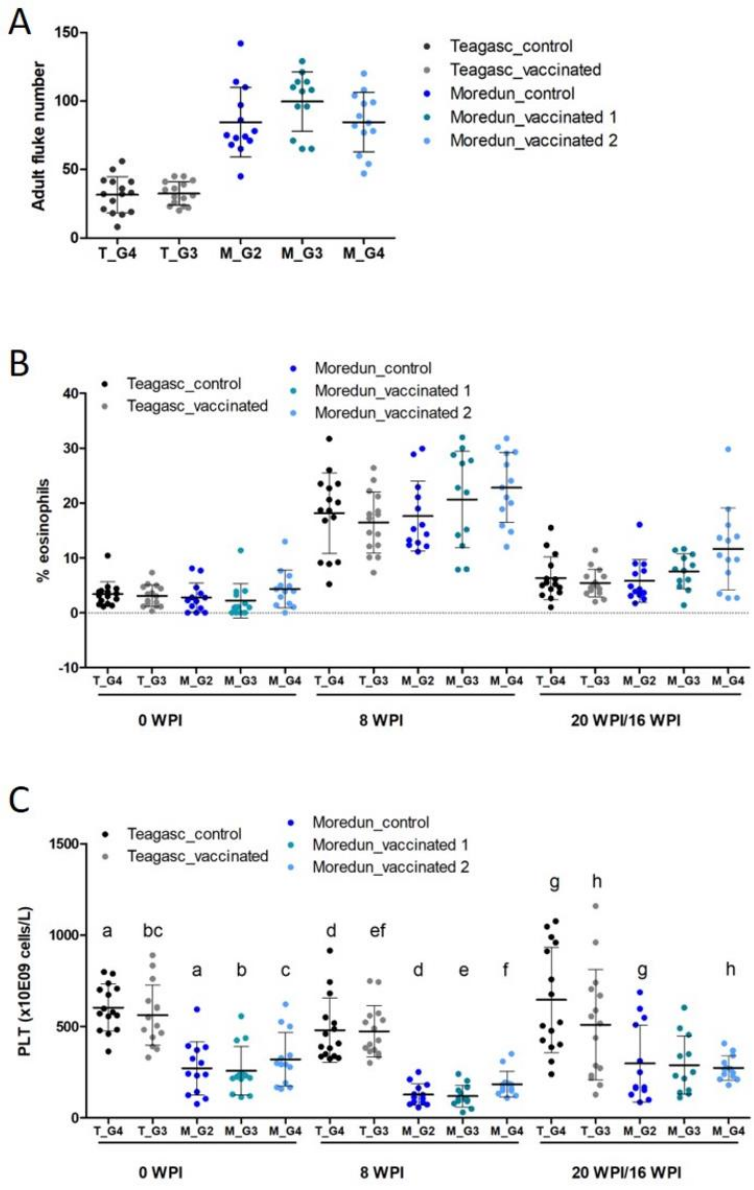
990

991 Figure 4



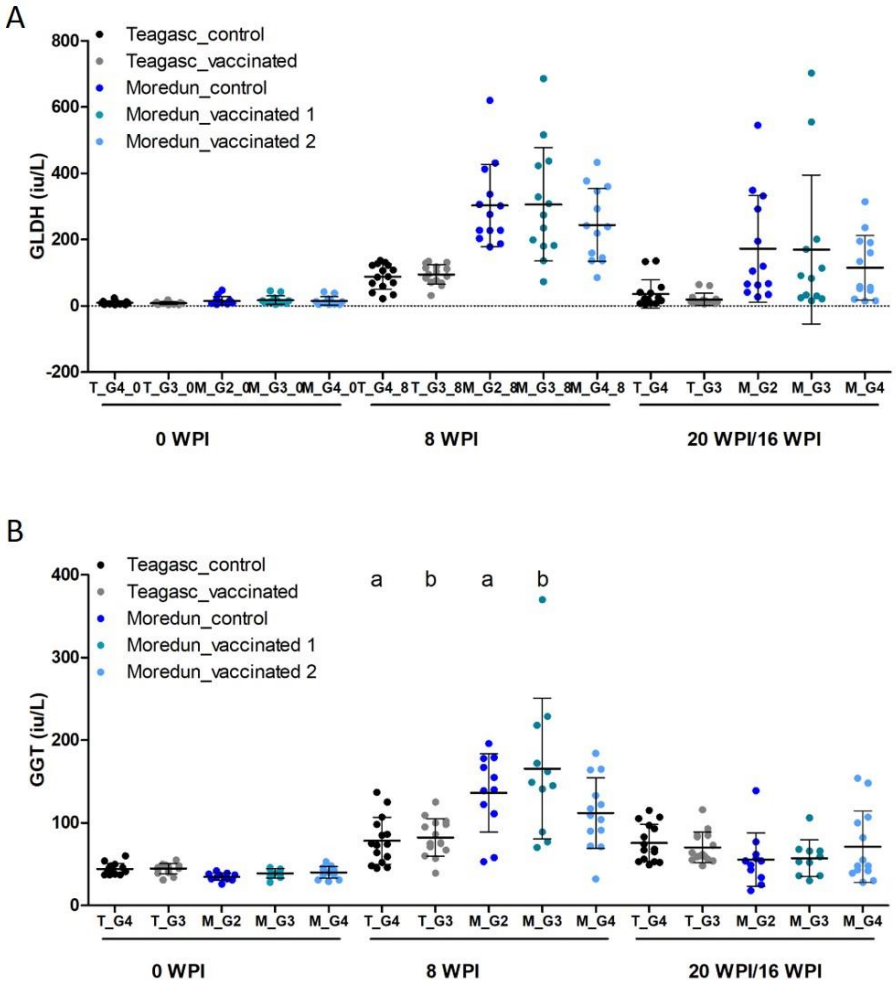
992

993 Figure 5



994

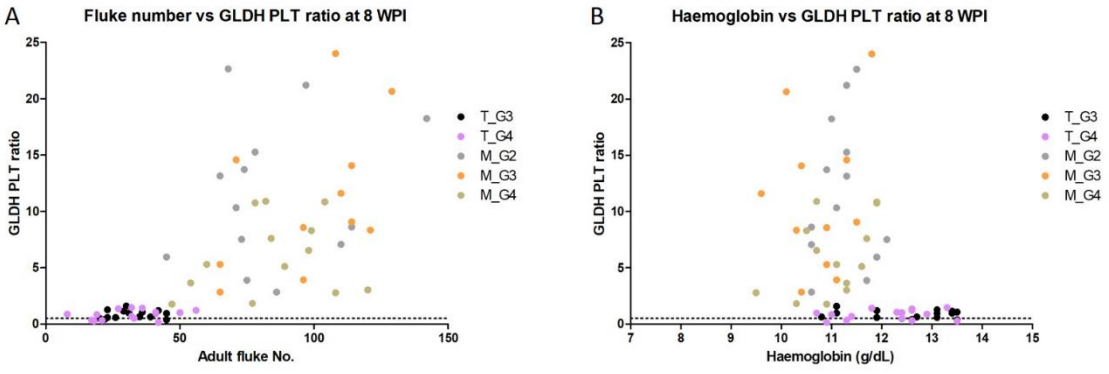
995 Figure 6



996

997

998 Figure 7



999

1000

1001