Title: Dairy heifers naturally exposed to *Fasciola hepatica* develop a type-2 immune response and concomitant suppression of leukocyte proliferation.

Running Title (max 54 characters): Bovine Immune Responses to *Fasciola hepatica*

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Abstract:

Fasciola hepatica is a parasitic trematode of global importance in livestock. Control strategies reliant on anthelmintics are unsustainable due to the emergence of drug resistance. Vaccines are under development, but efficacy is variable. Evidence from experimental infection suggest vaccine efficacy may be affected by parasite-induced immunomodulation. Little is known about the immune response to F. hepatica following natural exposure. Hence we analysed the immune responses over time in calves naturally exposed to F. hepatica infection.

Cohorts of replacement dairy heifer calves (n=42) with no prior exposure to F. hepatica, on three commercial dairy farms, were sampled over the course of a grazing season. Exposure was determined through F. hepatica-specific serum antibody ELISA and fluke egg counts. Concurrent changes in peripheral blood leukocyte sub-populations, lymphocyte proliferation and cytokine responses were measured. Relationships between fluke infection and immune responses were analysed using multivariable linear mixed effect models.

All calves from one farm showed evidence of exposure, whilst cohorts from the remaining two farms remained negative over the grazing season. A type-2 immune response was associated with exposure, with increased interleukin (IL)-4 production, IL-5 transcription and eosinophilia.

Suppression of parasite-specific PBMC proliferation was evident; while decreased mitogen stimulated IFN-γ production suggested immunomodulation, which was not restricted to parasite-specific responses. Our findings show that the global immune response is modulated towards a non-proliferative type-2 state following natural challenge with F. hepatica. This has implications for vaccination programmes in terms of the timing of administration of vaccination programmes, and for host susceptibility to co-infecting pathogens.

Introduction

Liver fluke (Fasciola hepatica) is a parasitic trematode of global importance capable of infecting a wide range of vertebrate hosts, including humans. Fasciolosis is considered a major issue for global
food security, with over 600 million sheep and cattle thought to be infected worldwide (1). In cattle, clinical presentation ranges from severe, acute to chronic disease depending on intensity and stage of infection, although both result in significant morbidity and/or production losses. Sub-clinical infections reduce weight gain, fertility and milk yield, which impacts on the economic viability of farm production systems (2, 3) and may last for months or years if untreated (4).

Both incidence and prevalence of infection has increased across Europe over the last decade. This is largely attributed to climatic changes, namely increases in ambient temperatures and rainfall, favouring the development of both *F. hepatica* and its intermediate snail host, *Galba truncatula*. As a result the range and prevalence of infection is increasing spatially and temporally, with changes in climate projected to further increase prevalence across Europe in the coming decades (5).

Control of fasciolosis in livestock is currently based on a limited number of anthelmintics, of which triclabendazole (TCBZ) has been the most heavily used due to its efficacy against both adult and migratory juvenile stages of the parasite (6). TCBZ resistant *F. hepatica* infections in livestock are now widely reported (7-12). There is therefore a need to develop novel approaches to control fasciolosis, with vaccination proposed as a potential adjunctive measure.

Current vaccine trials are focussed on several immunodominant parasite antigens including cathepsin L proteases, glutathione S-transferase (GST), fatty acid binding proteins (FABP) and leucine aminopeptidase (LAP), which have been tried in a number of formulations and host species (cattle, sheep and goats) in both native and recombinant forms with levels of protection ranging from 0-72% protection (13, 14). In cattle, as with other ruminant species, protection is often only partial with reductions in fluke burden, egg output and viability observed in vaccinated animals compared to unvaccinated controls (15, 16). In these circumstances, mathematical modelling has shown that such partial protection must be induced in at least 90% of the herd to have a meaningful impact on disease control (17).

Where vaccine-induced protection is demonstrated, it has been shown to correlate with parasite specific IgG2 isotype antibody titre and avidity and a reduction in arginase activity in CD14+ blood
monocyte derived macrophages, suggesting presence of a cell-mediated type-1 response is an important component of vaccine induced protection to *F. hepatica* (15, 18). Such responses, however, are not typically associated with infection in unvaccinated animals. Epidemiological evidence shows prevalence of *F. hepatica* infection increases with age (19) whilst chronically infected cattle remain susceptible to super-imposed experimental infections (20), suggesting an absence of any protective immunity. Studies investigating the immune responses of cattle experimentally infected with *F. hepatica* initially show a pro-inflammatory response progressing over the course of infection towards a polarised, non-proliferative state. Parasite-specific IL-2, IL-4 and IFN-γ production is described in hepatic lymph nodes at 10-14 days post infection (21), and parasite-specific IL-2 and IFN-γ production is also detected in peripheral blood mononuclear cells (PBMCs) from 1-3 weeks post infection (wpi), becoming absent by 5 wpi (22, 23). Similarly, mitogen and parasite-specific stimulated PBMC proliferation peaks around 2 wpi before returning to pre-infection levels (22-25). Thereafter, responses progress towards a non-proliferative state associated with type-2 cytokines, an IgG1 isotype antibody response and an eosinophilia (20, 24, 26). By 10-12 wpi, when the parasite has reached the bile ducts and matured, there is a lack of mitogen or parasite-specific lymphocyte proliferation and an upregulation in IL-10 and TGF-β (27, 28). It has been suggested that this fluke-induced modulation of the immune response facilitates its long term survival within the host (29), and may also impact on the host's susceptibility to other, co-infecting pathogens such as Salmonella Dublin (30) and *Mycobacterium bovis*, the causative agent of bovine tuberculosis (31, 32).

Current vaccine development programmes are based on the assumption that immune responses observed in experimentally infected cattle are representative of those in naturally infected cattle. Little however is known about the immune responses associated with naturally acquired *F. hepatica* infections, specifically the early stages of infection, and the extent to which parasite-induced immunomodulation is induced. This may have implications for how vaccines are delivered in the field, and for understanding how the parasite may affect host susceptibility to co-infecting
The aim of this study was therefore to define the immune responses in cohorts of dairy heifers on UK farms with typical management conditions and naturally exposed to challenge with *F. hepatica*.

**Materials and Methods**

Three commercial dairy farms were recruited into the study. They were identified as *F. hepatica* positive through bulk milk tank (BMT) antibody ELISA results (33) and positive composite faecal egg counts in adult cattle. On each farm, cohorts of replacement dairy heifer calves were recruited (n=42; 17, 17 and 8 animals from farms A, B and C, respectively) aged 90-377 days (mean=218.5, SD ±62.0). These animals had been housed since birth so had not been exposed to *F. hepatica*. This was confirmed prior to turnout by faecal fluke egg counts and anti-*F. hepatica* IgG serum antibody ELISA (34). Negative ELISA results indicated no maternally derived antibodies were detectable at turnout.

Animals were sampled monthly over the course of their first grazing season from turnout in spring (April-May) through to housing in autumn (October-November) 2013. On each occasion blood was collected via jugular venepuncture into plain and EDTA coated vacutainers and faecal samples were collected rectally. BMT samples were also taken at each visit to assess changes in level of exposure within the milking herd.

All the procedures used in this study were approved by the University of Liverpool’s Veterinary Research ethics committee (VREC100) and adhered to the conditions of the project license granted by the UK Home Office (HOL PPL40/3621). All farm data was stored in accordance with the UK Data Protection Act (1998).

*F. hepatica* serum and BMT IgG antibody ELISA

Tubes containing clotted blood were centrifuged at 2,000g for 5 min, serum collected, stored at 4°C and tested for fluke specific antibody within 5 days. A positive cut off of 20 Percent Positivity (PP) was used, giving a diagnostic sensitivity of 95% and specificity of 99% (34). BMT samples were
analysed using a positive cut-off value of ≥27PP, giving a diagnostic sensitivity of 96% and specificity of 80% as described previously (33).

**Fluke egg counts**

Faecal samples were stored at 4°C prior to analysis and examined for evidence of *F. hepatica* eggs following a standard sedimentation technique using 10g of faeces (35). This was performed on individual samples from the point of sero-conversion onwards, with counts done for every animal at the final time point irrespective of whether they had sero-converted. Nematode infections for all animals at each time point were assessed by faecal egg counts using the McMaster method with a sensitivity of 50 eggs per gram (epg) (36).

**Preparation of *F. hepatica* antigens**

Adult *F. hepatica* tegument and somatic antigen fractions for use in *in vitro* PBMC stimulation assays were prepared using previously described methods (23, 37); live adult fluke were collected from infected livers and incubated overnight to purge caecal contents, then washed three times in D-PBS (Sigma-Aldrich, St. Louis USA).

Tegument antigen (TegAg) was prepared by placing fluke in D-PBS with 1% Nonidet P-40 (BDH Chemicals, Poole UK), 1ml per fluke, and agitating for 1 hour at 4°C. Pierce® Detergent Removal spin columns (Thermo Fisher Scientific, Waltham USA) were used to remove the Nonidet P-40 detergent.

Somatic antigen (SomAg) was prepared from tegument-depleted fluke. These were washed in ice cold D-PBS and snap frozen overnight at -80°C. Fluke were then homogenised and diluted in D-PBS, 0.5ml per fluke, and left to settle overnight at 4°C. The supernatant was collected, centrifuged at 12,000g for 30 min at 4°C.

Both antigens were filter sterilised and shown to contain negligible levels of endotoxin at tissue culture concentrations (Thermo-scientific) (38). Protein concentrations were estimated using a Bradford assay (Thermo-scientific) and aliquots stored at -80°C.
Haematology, PBMC purification and Flow cytometry

Total and differential leukocyte counts were performed on EDTA-treated whole blood using a haemocytometer and thin blood smears, respectively, and used to calculate absolute counts for each leukocyte phenotype per ml of blood.

PBMCs were isolated from whole blood in a lateral flow hood using Optiprep™ (Sigma-Aldrich, St. Louis USA) following manufacturer’s recommendations. Optiprep™ was added to, and mixed with, EDTA treated whole blood, 1.3ml of optiprep per 10ml of blood, in a 50ml falcon tube onto which 1ml 20mM tricine-buffered saline was layered. Samples were centrifuged at 1,000g for 35 min at 20°C with the brake off following which the middle aqueous layer containing PBMCs was harvested and washed in PBS with 0.1% EDTA (Lonza, Basel Switzerland) to a maximum volume of 20ml.

Samples were centrifuged at 350g for 8 min at 20°C. Resulting supernatants were discarded and cell pellets re-suspended in 2ml of 0.9% NH₄Cl haemolysis buffer and gently agitated for 1 min at room temperature. Samples were then washed in 20ml PBS EDTA and centrifuged at 150g for 8 min at 20°C twice, with purified PBMCs then re-suspended and prepared for flow cytometry, proliferation and cytokine assays as described previously (67).

One colour indirect immunofluorescence labelling was performed on PBMCs as previously described (39), with lineage specific monoclonal antibodies to identify bovine CD4⁺ and CD8⁺ (40), WC1⁺ (41) and CD14⁺ subsets (42). Sub-populations of leukocytes were analysed using a MACSQuant® analyser (Miltenyi Biotech Ltd.). PBMCs were isolated through gating of forward and side scatter channels, with adjustments made using a post-hoc analysis template (MACSQuantify v.2.4.1221.1, Miltenyi Biotech Ltd.) to ensure appropriate fit for all samples. Leukocyte sub-populations were identified and quantified through FITC-positive fluorescence emission and used to calculate absolute counts per ml of blood.

Proliferation and cytokine measurements
PBMCs were adjusted to a concentration of 2 x 10^6 per ml in RPMI with 10% FCS and 100µg/ml penicillin and streptomycin and incubated in vitro with either ConA (5µg/ml), *F. hepatica* SomAg or TegAg (both 25µg/ml) or as unstimulated medium controls to assess proliferative and cytokine responses. For proliferation assays, 2 x 10^5 cells per well were cultured in triplicate in 96-well U-bottomed plates (Corning Life Sciences, Corning USA) for 5 days at 37°C, 5% CO₂. On the 5th day cultures were pulsed with 1µCi of [H³] tritiated thymidine (Perkin Elmer, Boston USA) for 5 hours then harvested onto glass filter mats and embedded in scintillation wax (Perkin Elmer, Boston USA). Beta-particle counts were measured with a MicroBeta² plate counter (Perkin Elmer, Boston USA). The stimulation index (SI) of mitogen/antigen stimulated cultures was calculated as the fold increase in emission counts compared to medium controls. If SI for the ConA positive control was <2, proliferation values for both mitogen and antigen stimulated cultures were excluded from further analysis.

For cytokine assays, PBMCs were incubated with ConA (5µg/ml), SomAg (25µg/ml) or as unstimulated medium controls for 48 hours at 37°C, 5% CO₂ in flat bottomed 24 well cell culture plates (VWR, Radnor USA). Upon completion, culture supernatants were removed and stored at -20°C with PBMCs stored separately at -20°C in RNAlater (Sigma-Aldrich, St. Louis USA).

**Cytokine production and transcription assays**

Cytokine production and transcription was measured in animals that sero-converted (farm A). Interferon (IFN)-γ and IL-4 concentrations were measured in supernatants using commercially available ELISAs following manufacturer’s protocols (MCA5638KZZ & MCA5892KZZ respectively, AbD Serotec, Raleigh USA). IL-10 production was measured in SomAg-stimulated and medium control cultures using a previously validated sandwich ELISA (43).

Paired samples were used to investigate difference in TGF-β production in early versus chronic infection; samples for each individual animal were selected at the closest available time-point to sero-conversion to represent early infection, and from the last available time point to represent
Chronic stages of infection. Bio-active bovine TGF-β was measured in SomAg-stimulated and medium control cultures using a commercially available ELISA kit according to manufacturer’s recommendations (Promega, Madison USA) (44). Quantitative real-time (q)PCR was used to measure IL-2 and IL-5 mRNA against 28s housekeeper gene transcription in cultured PBMCs using previously published primers (NCBI accession No. AF154866, M12791 and EU915048.1, respectively). RNA extraction was performed using the RNeasy Mini kit (QIAGEN, Limburg Netherlands), and mRNA quantified with RiboGreen® (Invitrogen Life Technologies, Grand Island USA). Genomic DNA digestion and cDNA synthesis was then performed on 1μg of mRNA template for each sample using Quantitect Reverse transcriptase kit (QIAGEN, Limburg Netherlands). qPCR analysis was performed using a standard protocol with SYBR Green (Bioline reagents Ltd., London, UK) as described previously (45). Samples were assayed alongside known concentration standards diluted in a 10-fold series in 100ng/μl yeast tRNA (Invitrogen Life Technologies, Grand Island USA) to prevent aggregation. Reactions were performed using a DNA Engine opticon 2 continuous Fluorescence detector. A final melting curve analysis was performed from 50-95°C to confirm the specificity of the amplification products. Sample copy numbers were determined using linear regression of standard concentrations following adjustment of Ct cut-off values to the log-linear phase of amplification. Results for IL-2 and IL-5 transcription are shown as relative expression against the 28s housekeeper gene (per million copies).

Statistical Analysis
Data analysis was performed using multivariable linear mixed effects models in the “nlme” package (46) in the R statistical software environment (47). Response variables (Y) chosen as indicators of exposure to *F. hepatica* were antibody PP value (Ab) to assess responses over the course of infection, and change in PP values (ΔAb) used to examine immunological parameters associated with the early stages of infection. This approach was taken as whilst PP values (Ab) increased over the
course of the study (Figure 1), the greatest changes in PP values (ΔAb) were seen at or soon after sero-conversion (Figure 2).

Details of the structure of each statistical model are shown in (Table 1). For all models, leukocyte counts, PBMC proliferative responses, farm location, animal age, weight and days exposure (days at pasture) were modelled as fixed effects (Table 1), with individual animal identity modelled as a random effect to account for the increased relatedness of responses measured in the same animal.

Models containing data from the fluke infected cohort (farm A) only (Table 1; lme_2 & 4) were analysed to allow the inclusion of the ex vivo PBMC cytokine responses measured as explanatory variables, thereby giving a more in-depth analysis of the immune responses present in these animals. Raw immunological data are summarized in supplemental material (Figures S1-6).

Three models were produced using Ab as the response variable (Table 1; lme_1, 2 & 3). The first analysis (lme_1) included all data for all three farms over the 7 month sampling period. Model lme_2 included data from the fluke infected cohort (farm A) only. Model lme_3 showed the analysis of data from fluke negative cohorts (farms B & C) only.

When analysing immune responses associated with ΔAb, data collected pre- and post-seroconversion was considered separately to allow the small changes in PP value associated with pre-seroconversion and late stage infection to be considered separately. Consequently, two models were produced using (ΔAb) as the response variable (Table 1; lme_4 & 5). The model lme_4 used post sero-conversion data from the fluke positive cohort (farm A) to measure changes in immune response associated with early exposure, whilst the model lme_5 used sero-negative data from farms A, B & C.

Models containing only data from sero-negative measurements (lme_4 & 5) served to investigate potential physiological and/or age related changes, thus avoiding incorrectly attributing such observations to *F. hepatica* exposure.

Prior to the linear mixed model analysis, the relationship between response and fixed effect explanatory variables was assessed, using a Box-Cox transformation (48) with power \( \lambda \) determined.
by maximum likelihood analysis used to transform the response variable to ensure an appropriate linear relationship (Table 1) (49).

For models with Ab as the response variable (Y), an autoregressive correlation matrix of order 1 was also used to account for the relatedness of measurements resulting from repeated sampling, and in particular the fact that measurements taken close together in time are likely to be more similar than those taken further apart.

Model refinement was then carried out using a stepwise Akaike information criterion (AIC) selection method (50) using complete case data for all models being compared with maximum likelihoods (ML) using the “MASS” package. The criterion of a reduction in AIC of 2 or greater was deemed indicative of model superiority (Table 1). The selected model was then fitted to all data for which complete observations were available using restricted maximum likelihoods (REML). This final model was checked for goodness of fit and normality using residual analysis and qq-plots, respectively (Figures S7-8). Missing (NA) values were considered to be “dropouts completely at random” (DCAR) since animals were moved between management groups throughout the grazing season for unrelated reasons (eg. stocking density, pasture management etc.) (51).

Model outputs were interpreted by inspecting the coefficient (β) and associated standard error for each explanatory variable (x) included in the final model. The estimated coefficients indicated whether a positive or negative relationship was present between each explanatory variable (x) and the (transformed) response variable (Y), once all other explanatory variables present in the analysis had been taken into account.

To assess TGF-β expression in early versus late stage infection, results were analysed using two-tailed paired T-tests. Differences in measurement between medium control and SomAg stimulated PBMC cultures for each time point were analysed using unpaired two-tailed T-tests.

Results

1. Faecal egg counts and antibody responses
All calves remained healthy throughout the study, with no clinical signs observed as a result of *F. hepatica* infection, nematode burden or other disease. On each farm the adult milking herd was monitored monthly using a sample of milk from the bulk tank. All three herds tested positive on every occasion with BMT antibody values ranging from 33-50PP, 54-61PP and 79-114PP for farms A, B and C, respectively.

Sero-conversion was observed in all 17 calves on farm A; PP values increased over the course of the study, reaching 37-98 PP at the final time point (Figure 1). Fluke eggs were detected in 10 of the 17 animals from farm A by the final time point, with counts in all cases less than 1 egg per gram of faeces. Paramphistome eggs were also observed in low numbers in seven calves at the final time point. Six of these were positive for both *F. hepatica* and paramphistome eggs.

PP values for the cohorts from farms B and C remained negative throughout the study except for two animals from farm B that had positive PP values at a single time point. These PP values were close to the cut off (20.3 & 24.7) and samples taken from these two calves on every other occasion were negative suggesting these results were false positives. All calves from farms B and C remained negative for fluke eggs for the duration of the study.

Low numbers of nematode eggs were detected in calves on all three farms. Only two positive samples, with counts of 50 epg were detected on farm A at a single time point. Both trichostrongyle and *Nematodirus* spp. eggs were observed intermittently in 15/17 calves from farm B from August onwards, and trichostrongyle eggs were observed intermittently in 4/8 calves from farm C from July onwards.

### 2. Immunological correlates of infection

Twenty three immune and four signalment parameters were measured monthly for each calf, yielding over 1100 separate data points. To analyse these data we used multivariable linear mixed effect modelling. Two response variables were used: 1) antibody PP value (Ab) was used to indicate infection progression and 2) change in antibody (ΔAb) after sero-conversion was used to indicate the
point of first exposure and early stage of infection. ΔAb was used because the increase in PP values were greatest at the point of sero-conversion (Figure 2).

i. Association between Ab and immune responses

The output from the three models using Ab as the response variable (lme_1, 2 & 3) are shown in Table 2. Statistically significant positive associations were found in all three models between Ab (response variable) and days of exposure, showing that the longer the calves were grazing, the greater the likelihood of infection. A significant negative relationship between Ab and SomAg-specific PBMC proliferation was also observed in all three models.

When data from all three farms were used (lme_1), statistically significant negative associations were found for farms B and C relative to farm A. Statistically significant positive associations were detected for eosinophil and CD8⁺ PBMC counts and a negative coefficient was estimated for peripheral blood WC1⁺ counts.

When the immune response data from farm A were used (lme_2), there was a negative association between Ab and ConA-induced IFN-γ production, and a positive association with ConA-induced IL-4 and SomAg-induced IL-5 transcription.

The model with data from fluke-negative cohorts on farms B & C (lme_3) showed a significant increase in peripheral CD8⁺ T-cell counts and corresponding decrease in CD4⁺ T-cell counts.

ii. Association between ΔAb and immune responses

When change in PP value (ΔAb) was used to indicate time of exposure (Table 3; lme_4), there was a significant negative association between ΔAb and days of exposure, suggesting infection occurred early after turnout and that ΔAb is a good measure of early infection. A negative association was also observed for SomAg-specific PBMC proliferation, whilst a positive associations were observed for TegAg-specific PBMC proliferation, ConA stimulated IL-4 production and SomAg stimulated IL-5 transcription.
When using sero-negative data from all farms (Table 3; lme_5), there was a significant negative association with SomAg-specific PBMC proliferation, and a positive association with peripheral blood CD8\(^+\) counts.

3. **Cellular, proliferation and cytokine responses**

Eosinophil counts increased in all the animals on all three farms over the course of the study (Figure 3). PBMC proliferation responses were variable both between animals and for individual animals at different time points. The highest parasite-specific proliferation responses were observed in animals from farm A. Similar variation was observed in the cytokine responses of animals from farm A. An increase in ConA-stimulated IFN-\(\gamma\) production was observed following sero-conversion and there was evidence of ConA stimulated IL-4 production also. No IFN-\(\gamma\) production was detected in response to stimulation with SomAg, whilst only low levels of IL-4 production were observed. IL-2 and IL-5 transcription was similarly variable between and within individuals over the course of the study in response to stimulation with both ConA and SomAg.

No significant difference was found between levels of TGF-\(\beta\) expression in early versus late stage infection in either medium control or SomAg stimulated PBMC cultures (\(p=0.791 & 0.828\), respectively), nor was any significant difference found between TGF-\(\beta\) levels in medium control or SomAg stimulated cultures at either time point (\(p=0.291 & 0.306\), respectively).

**Discussion**

In this study, we have demonstrated that dairy calves develop a polarised, non-proliferative type-2 response following primary natural challenge with *F. hepatica*. Studies using experimentally infected calves have described an initial inflammatory response comprised of mixed cytokine (IFN-\(\gamma\) and IL-4) production and antigen specific proliferation in PBMC cultures, which subside from 4-6 weeks post-infection. Thereafter parasite-specific IgG1 and IL-4 responses are detected indicating a polarisation.
towards a type-2 immune response as the infection progresses \( (20, 22, 23, 52) \). Our analysis of naturally infected calves show an association with increased eosinophil counts, IL-4 production, IL-5 transcription and decreased IFN-\( \gamma \) production, indicating polarisation towards a type-2 response as infection progresses. Furthermore, the increased IL-4 and decreased IFN-\( \gamma \) production by ConA-stimulated PBMCs suggests polarisation of the global T-cell response is present. This is consistent with findings in experimentally infected cattle \( (32, 53) \). The presence of a generalised type-2 immune environment may help explain why calves are less able to respond effectively to co-infecting pathogens. For example, cattle infected with \( F. hepatica \) are more susceptible to Salmonella Dublin infection \( (30, 32) \).

Our findings differ from those obtained from experimentally infected calves however, in the rate and stage at which immune modulation is observed. Experimentally infected cattle have increased IL-2 and IFN-\( \gamma \) production in the first 2-3 weeks post infection \( (22, 23) \), whilst our study suggests the early stages of natural infection are associated with increased IL-4 production and IL-5 transcription.

Similarly, D. G. Clery and G. Mulcahy \( (22) \) showed parasite specific proliferation within 2 to 3 weeks post infection in experimentally infected calves, whilst our study detected a significant negative association between SomAg specific PBMC proliferation at all stages of infection. Early proliferative responses were observed in only 3/17 animals. For this study, samples were taken monthly, hence some transient early pro-inflammatory responses may have been missed. Nonetheless, our findings suggests that, unlike experimental infections, polarisation of the immune response in naturally-acquired infection is present from very early in infection.

These differences may relate to the infectious challenge administered in experimental infections compared to those encountered under field conditions. Most experimental infections have used either a single dose, or ‘trickle’ type infection, where boluses of several hundred, up to 1000 metacercariae are administered per dose \( (26, 54) \). The epidemiology of \( F. hepatica \) in temperate regions typically results in small numbers of metacercariae present on pasture early in the spring followed by increasing numbers of metacercariae appearing on pasture towards the end of the
grazing season (55). Hence administering a large infectious challenge to immunologically naïve cattle may not be representative of normal field conditions and may result in a more profound innate cellular response during the early stages of experimental infection.

Our results suggest that challenge at the start of the grazing season was not associated with detectable pro-inflammatory or proliferative responses. The immune-modulation induced during this primary challenge also may prevent any subsequent pro-inflammatory, proliferative responses developing later in the season when animals are exposed to a more substantial infectious challenge as pasture contamination increases. These findings have implications for vaccine programmes, since current efforts are focussed on enhancing cell-mediated type-1 responses (15, 18). Our results suggest the efficacy of such vaccines would be negatively impacted if administered following natural exposure to *F. hepatica* under field conditions. These vaccines would therefore need to be administered and be fully effective prior to any exposure to infection.

In experimentally infected calves, a systemic regulatory response develops during the chronic stages of infection characterised by increased parasite specific IL-10 and TGF-β production by PBMC (27). In this study, we found no significant association between infection status and parasite-specific IL-10 or TGF-β production by PBMCs. Again, this may relate to differences in the way in which the calves were exposed to infection, although it is possible that such regulatory responses were present in local and regional lymph nodes, as this has been observed previously in cattle and sheep harbouring naturally acquired, chronic *F. hepatica* infections (28, 56).

Eosinophilia is a feature of many helminth infections. Calves on farm A were treated with three doses of ivermectin over the course of the grazing season, and nematode egg counts remained at or close to zero throughout the study period. The eosinophilia observed in this cohort was most likely to be in response to *F. hepatica* rather than nematode infection, particularly since there was a significant positive relationship between fluke Ab PP value and both peripheral eosinophil counts and SomAg-stimulated IL-5 transcription. This is consistent with previous findings of eosinophilia in *F. hepatica* infected cattle (26, 57).
Both fluke infection and specific fluke antigens are known to have immuno-suppressive and/or modulatory effects on both the innate and adaptive immune responses (20, 27, 52, 58). Differences between the relationships of SomAg and TegAg PBMC proliferation and the early stages *F. hepatica* exposure (ΔAb) may be the result of differences in composition of these two antigen fractions and their functions *in vivo*. Overall, however, our results show that in naturally infected calves, there is an absence and/or suppression of parasite-specific T cell proliferation.

The negative relationship between Ab and WC1⁺ PBMC counts is most likely to be due to age and maturation of the immune system. WC1⁺ γδT-cells are known to decrease in number in the peripheral blood as cattle mature. WC1⁺ cell populations constitute approximately 15% of PBMCs in calves aged 3-12 months in age, decreasing to around 5% by 3 years of age (59). In our study, animals ranged in age from 90-377 days at the beginning and were 310-587 days of age by the end. The respective mean WC1⁺ PBMC counts were 20% (6-32%) and 6% (0.5-24%). Similarly, the positive relationship between Ab and CD8⁺ PBMC counts may also signify the normal maturation of the immune system rather than a direct impact of infection since this was also observed in uninfected animals.

In spite of evidence from the adult milking herd that *F. hepatica* infection was present on all three farms, the calves on farms B and C showed no evidence of infection. In both cases these calves were kept in different parts of the farm and did not share pastures with the adult cows. In contrast the cohort on farm A was managed in a rotational grazing system that included pastures grazed by adult cattle. This suggests the fields used for calves on farms B and C had no habitat suitable for the intermediate host, *Galba truncatula* and were not contaminated with metacercariae.

In this study, multivariable linear mixed-effect regression models were used to analyse a longitudinal set of data from cattle naturally exposed to *F. hepatica*. This analysis was useful since it allowed the simultaneous assessment of multiple immunological variables to identify the key parameters associated with infection. To the best of our knowledge this is the first time such techniques have
been used to analyse complex immunological responses to natural infection in livestock and may represent a potentially useful template for future studies.

In conclusion, through analysis of a longitudinal dataset using multivariable linear mixed-effect regression analysis we have demonstrated that natural exposure to *F. hepatica*, dairy heifers results in a generalised type-2 immune state with concomitant suppression of proliferation responses. This has significance for both vaccine administration programmes and susceptibility to other common co-infecting pathogens of livestock.

Acknowledgments

JGB, DW, MB and AK were responsible for conception and design of work, JGB, CH and HC were responsible for acquisition, analysis and interpretation of data. All contributing authors were responsible for drafting and/or revision of intellectual content and approval of the submitted manuscript and accept accountability for the accuracy and integrity of the work herein.

Additionally, we would like to thank Dr Jayne Hope (Roslin institute, Edinburgh, UK) for the kind donation of the anti-bovine IL-10 ELISA standards used in this study, Dr Ben Makepeace for his advice and guidance in the use of the qPCR techniques described and the study farmers for their cooperation and willingness to be involved in our investigation. This study was funded by the EU consortium PARAVAC (FP7-VKBBE-2010-4-265862) awarded to DJLW.

References


Table 1: Linear mixed effects model structures. Response variable (Y) indicates the subject of each respective model. Box-Cox value (λ) is the optimal transformation factor for the response variable to ensure linear fit to the proposed fixed-effect variables. Where nil is quoted, transformation was not required. For initial fixed-effect explanatory variables "Leuk. counts" refers collectively to leukocyte count data (eosinophils (Eo), neutrophils (No) and CD4⁺, CD8⁺, WC1⁺ & CD14⁺ PBMCs) per ml of peripheral blood, "PBMC prolifn." to PBMC proliferation data (ConA, SomAg and TegAg stimulated cultures), whilst "PBMC cytokines" refers to all available PBMC cytokine production and transcription data (IFN-γ, IL-4 and IL-10 production, IL-2 & IL-5 relative gene transcription). For cytokine data present as final explanatory variables square parentheses [ ] indicate the culture conditions present; medium control (Med), ConA, SomAg or TegAg. Time series plots of the immunological data included in these models are included as supplementary material.

<table>
<thead>
<tr>
<th>Linear mixed effect model</th>
<th>Response variable (Y)</th>
<th>Box-Cox value (λ)</th>
<th>Data analysed</th>
<th>Initial fixed-effect explanatory variables (x); [AIC value]</th>
<th>Final explanatory variables (x) following stepwise AIC selection; [AIC value]</th>
</tr>
</thead>
<tbody>
<tr>
<td>lme_1</td>
<td>Ab</td>
<td>-0.2</td>
<td>All data</td>
<td>Farm + Days exposure + Age + Weight + Leuk. counts + CD4:CD8 ratio + PBMC prolifn.; [AIC = 41.98]</td>
<td>Farm + Days exposure + Eo + CD4 + CD8 + WC1 + SomAg prolifn. + TegAg prolifn.; [AIC = 37.99]</td>
</tr>
<tr>
<td>lme_2</td>
<td>Ab</td>
<td>0.2</td>
<td>Farm A</td>
<td>Days Exposure + Age + Weight + Leuk. counts + CD4:CD8 ratio + PBMC prolifn. + PBMC cytokines; [AIC = 166.04]</td>
<td>Days Exposure + Eo + SomAg prolifn. + IFNγ [ConA] + IFNγ [SomAg] + IL-4 [ConA] + IL-10 [Med] + IL-10 [SomAg] + IL-2 [ConA] + IL-5 [SomAg]; [AIC = 145.57]</td>
</tr>
<tr>
<td>lme_3</td>
<td>ΔAb</td>
<td>nil</td>
<td>Sero-positive values; Farm A</td>
<td>(see lme_2); [AIC = 354.80]</td>
<td>Days exposure + CD4 + WC1 + SomAg prolifn. + TegAg prolifn. +IL-4 [ConA] + IL-5 [SomAg]; [AIC = 329.74]</td>
</tr>
<tr>
<td>lme_4</td>
<td>Ab</td>
<td>-0.2</td>
<td>Farms B &amp; C</td>
<td>(see lme_1); [AIC = 38.29]</td>
<td>Farm + Days exposure + SomAg prolifn + Eo + CD4 + CD8 + WC1; [AIC = 26.06]</td>
</tr>
<tr>
<td>lme_5</td>
<td>ΔAb</td>
<td>nil</td>
<td>Sero-negative values; Farms A, B &amp; C</td>
<td>(see lme_1); [AIC = 653.12]</td>
<td>Farm + CD4 + CD8 + CD4:CD8 ratio + SomAg prolifn. + TegAg prolifn.; [AIC = 640.33]</td>
</tr>
<tr>
<td>lme_6</td>
<td>ΔAb</td>
<td>nil</td>
<td>Farms B &amp; C</td>
<td>(see lme_1); [AIC = 580.03]</td>
<td>Eo + CD8; [AIC = 561.09]</td>
</tr>
</tbody>
</table>
Table 2: Multivariable linear mixed effect model outputs for anti-*F. hepatica* antibody PP value (Ab) as response variable (Y). Statistically significant (p<0.05) explanatory variables (x) are shown with corresponding coefficient (β) and standard error (SE) values to describe their respective relationships with Ab. Values rounded to 3 decimal places, or 1 significant figure.

<table>
<thead>
<tr>
<th>Model ID</th>
<th>Data used</th>
<th>Explanatory variable (x)</th>
<th>Coefficient value (β)</th>
<th>Standard error (SE)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>lme_1</td>
<td>All data</td>
<td>Farm B</td>
<td>-0.687</td>
<td>0.077</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Farm C</td>
<td>-0.874</td>
<td>0.087</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Days exposure</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EF/ml</td>
<td>0.0001</td>
<td>0.00004</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8/ml</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WC1/ml</td>
<td>-0.0001</td>
<td>0.00004</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SomAg</td>
<td>-0.006</td>
<td>&lt;0.003</td>
<td>0.024</td>
</tr>
<tr>
<td>lme_2</td>
<td>Farm A</td>
<td>Days exposure</td>
<td>0.027</td>
<td>0.003</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(positive cohort)</td>
<td>SomAg</td>
<td>-0.012</td>
<td>0.005</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[ConA] IFNγ</td>
<td>-0.0001</td>
<td>&lt;0.00006</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[ConA] IL-4</td>
<td>0.0005</td>
<td>0.0002</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[SomAg] IL-5</td>
<td>0.003</td>
<td>0.001</td>
<td>0.012</td>
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<tr>
<td>lme_3</td>
<td>Farms B &amp; C</td>
<td>Day exposure</td>
<td>-0.201</td>
<td>0.086</td>
<td>0.029</td>
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<tr>
<td></td>
<td>(negative cohorts)</td>
<td>CD4/ml</td>
<td>-0.0001</td>
<td>0.00004</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td></td>
<td>CD8/ml</td>
<td>0.0004</td>
<td>0.0001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SomAg</td>
<td>-0.007</td>
<td>0.003</td>
<td>0.042</td>
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</table>
Table 3: Multivariable linear mixed effect model outputs for change in antibody PP value (ΔAb) as response variable (Y). Statistically significant (p<0.05) explanatory variables (x) are shown with the coefficient (β) and standard error (SE) values to describe their respective relationships with ΔAb. Values rounded to 3 decimal places.

<table>
<thead>
<tr>
<th>Model ID</th>
<th>Data used</th>
<th>Explanatory variable (x)</th>
<th>Coefficient value (β)</th>
<th>Standard error (SE)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>lme_4</td>
<td>Farm A (post sero-conversion)</td>
<td>Days exposure</td>
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<td>0.107</td>
<td>0.004</td>
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<tr>
<td></td>
<td></td>
<td>SomAg</td>
<td>-2.794</td>
<td>1.211</td>
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<tr>
<td></td>
<td></td>
<td>TegAg</td>
<td>2.254</td>
<td>1.064</td>
<td>0.049</td>
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<td></td>
<td></td>
<td>[ConA] IL-4</td>
<td>0.012</td>
<td>0.050</td>
<td>0.033</td>
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<tr>
<td></td>
<td></td>
<td>[SomAg] IL-5</td>
<td>0.071</td>
<td>0.029</td>
<td>0.026</td>
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<tr>
<td>lme_5</td>
<td>Farms A, B &amp; C (Sero-negative values)</td>
<td>Farm B</td>
<td>-12.009</td>
<td>4.675</td>
<td>0.015</td>
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<tr>
<td></td>
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<td>Farm C</td>
<td>-14.760</td>
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<td></td>
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<td>0.009</td>
<td>0.008</td>
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<td>SomAg</td>
<td>-0.621</td>
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<td>0.027</td>
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<tr>
<td></td>
<td></td>
<td>TegAg</td>
<td>0.712</td>
<td>0.248</td>
<td>0.005</td>
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</tbody>
</table>
Figure 1: Antibody PP values of individual animals over the study period for the three dairy farms (A, B & C). Diagnostic positive cut-off value (PP=20) is denoted by horizontal line. Month of sampling denotes the time point for each sequential visit to each farm.

Figure 2: Change in antibody PP value (ΔAb) over the course of infection for farm A. Positive values indicate an increase in Ab PP value from one time point to the next, and negative values denote a decrease in Ab PP value compared to the previous month. Values from 1 month prior to seroconversion (-1) are considered separately from other sero-negative values (-ve) as infection may have been present at this time point but the animals had not seroconverted. In experimentally infected animals, seroconversion occurs 2-4 weeks post-infection (34).

Figure 3: Peripheral blood eosinophil counts for individual animals over the study period for the three dairy farms (A, B & C). Month of sampling denotes the time point for each sequential sampling visit.