

# Host responses during experimental infection with *Fasciola gigantica* or *Fasciola hepatica* in Merino sheep

## I. Comparative immunological and plasma biochemical changes during early infection

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### Abstract

This study reports the early biochemical changes in plasma, comparative host-immune responses and parasite recovery data in Merino sheep during the first 10 weeks of infection with *Fasciola gigantica* and *Fasciola hepatica*. One group of sheep were uninfected, four groups of sheep received incremental challenge doses of *F. gigantica* metacercariae (50, 125, 225 and 400, respectively) and the sixth group was challenged with 250 *F. hepatica* metacercariae. At 10 weeks post infection (wpi), sheep challenged with *F. hepatica* showed the greatest fluke recovery (mean 119, range 84–166); a significantly higher biomass of parasites recovered (2.5-fold greater than the highest dose of *F. gigantica*); and a greater mean % parasite recovery (39.3%, range 27–55%) than any group challenged with *F. gigantica*. Within the groups dosed with *F. gigantica* a strong dose-dependent response was observed in both fluke recovery and fluke biomass with increasing dose of metacercariae. The mean % parasite recovery of *F. gigantica* infected groups 1–5 were 26, 23, 26 and 25%, respectively, suggesting a uniform viability of parasite establishment independent of infection dose. At 6 wpi, elevated levels of plasma GLDH were observed in the *F. gigantica* infected groups compared to the uninfected sheep ( $p < 0.005$ ) whereas the *F. hepatica* challenged group had four-fold higher levels of GLDH compared to the *F. gigantica* infected group ( $p < 0.001$ ). Elevated levels of GGT as an indicator of epithelial damage in the bile duct was only seen in the group challenged with *F. hepatica* at 10 wpi when it rose from below 100 IU/l to approximately 250 IU/l ( $p < 0.0001$ ) whereas no detectable increase in GGT was observed in any of the groups challenged with *F. gigantica*. The white blood cell response to *F. hepatica* infection was biphasic with the initial peak at 4 wpi and a second peak at 9 wpi, corresponding to the period of migration of juvenile fluke in the liver and the time when adult flukes are migrating into the bile duct, respectively. This biphasic response was also evident in the changes in the eosinophil counts and serum haemoglobin levels. There was a trend toward higher parasite-specific IgG<sub>2</sub> titres in sheep infected with lower worm burdens, suggesting that higher *F. gigantica* or *F. hepatica* burdens suppress IgG<sub>2</sub> responses. The findings of this study suggest that, in early infection in a permissive host, *F. hepatica* appears to be more pathogenic than *F. gigantica* because of its rapid increase in size and the speed of its progression through the migratory phases of its life cycle.

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## 1. Introduction

Fasciolosis (infection with *Fasciola gigantica* or *Fasciola hepatica*) is a major parasitic disease of livestock with over 700 million production animals at risk of infection and worldwide economic losses estimated at >US\$3.2 billion pa (Spithill et al., 1999). In temperate regions *F. hepatica* commonly infects sheep and cattle while, in tropical regions, *F. gigantica* infects sheep, cattle and buffalo.

Many sheep breeds are susceptible to *Fasciola* infection including most sheep selected for enhanced production traits such as wool production in Merino sheep. However, in contrast to *F. hepatica*, it has been demonstrated that some sheep breeds are resistant to infection by *F. gigantica* (A'Gadir et al., 1987; reviewed in Spithill et al., 1999). High resistance to *F. gigantica* infection has been observed in Indonesian Thin Tail (ITT) sheep (Wiedosari and Copeman, 1990; Roberts et al., 1997a,b,c). ITT sheep express resistance to *F. gigantica* within the prepatent period of a primary infection and acquire a higher level of resistance after exposure (Roberts et al., 1997a,c). In addition, numerous studies have shown that sheep do not acquire resistance to a secondary *F. hepatica* infection (Boyce et al., 1987; Chauvin et al., 1995; Roberts et al., 1997a; Piedrafito et al., 2004).

These findings suggest that *F. hepatica* and *F. gigantica* differ in some fundamental biological trait(s) which renders *F. gigantica* more susceptible to immune effector mechanisms (Spithill et al., 1997; Piedrafito et al., 2004). However, the relative rates of development of the *F. gigantica* and *F. hepatica* parasites, when compared to the development of host response factors, is poorly understood. A comparison of the humoral response during infection of Belle Islois sheep to *F. hepatica* and *F. gigantica* (Zhang et al., 2004) as well as the modulation of lymphocyte and eosinophil responses (Zhang et al., 2005a,b) suggested that sheep are less susceptible to *F. gigantica* compared with *F. hepatica*. Although studies have suggested that the host immune response to the parasite plays an important role in the susceptibility of sheep to *Fasciola* spp. (Wiedosari and Copeman, 1990; Roberts et al., 1997a,b,c; Piedrafito et al., 2004) it has been shown that parasite modulation of the immune response may also play a role in immune evasion and subsequent host susceptibility (Zimmerman et al., 1983; Chauvin et al., 1995; Prowse et al., 2002; Zhang et al., 2005a,b).

There are numerous studies of sheep breeds infected with *F. hepatica*, but few on the biological and immunological responses of sheep to *F. gigantica*

infection. In addition, there are almost no comparative studies on the immuno-biology of host responses to infection with a concurrently performed challenge with *F. gigantica* or *F. hepatica* in sheep of a similar genetic background and identical rearing practices (Zhang et al., 2004, 2005a,b). Here, we report physiological changes and comparative host immune responses in Merino sheep during early infection with *F. gigantica* or *F. hepatica*. This is the first direct comparative report of the early biochemical and immunological changes in sheep challenged with these parasites. A better understanding of the immune response to both *F. gigantica* and *F. hepatica* would be helpful in the development of rational control strategies of these parasites.

## 2. Materials and methods

### 2.1. Experimental animals

Fifty-nine young (6 months old) Merino wethers were purchased from areas in NSW classified as low risk for *F. hepatica* and located on site (University of Sydney, Camden campus) 3–4 weeks before commencement of the experiment. The Merino is a highly valued commercial sheep breed and was chosen for the comparative infections since this breed is susceptible to infection by both *F. hepatica* and *F. gigantica*. The naivety of the animals was confirmed by a negative reaction in an ELISA and a Western blot using *Fasciola* whole worm extract as antigen (Estuningsih et al., 1997). The sheep were treated for nematode parasites by drenching with Ivomec at the recommended dose rate. Sheep were introduced to a commercial pelleted sheep ration consisting of 13% protein, 11.5 MJ ME/kg energy. Following stabilisation of feed intake, sheep were given *ad libitum* access to feed and water. Sheep were weighed weekly, 2 weeks prior to and during the experimental period. All sheep were allocated to 1 of 6 experimental treatment groups based on stratified sampling following ranking on body weight at time of challenge, and held in group pens of 10 sheep/group in groups 1–5 and 9 sheep/group in group 6 for the duration of the experiment (Table 1). Animal ethics guidelines were strictly adhered to in the care of the animals.

### 2.2. Parasites, parasite extracts and reagents

Metacercariae for infections were obtained from infected *Lymnaea rubiginosa* snails collected at Surade, West Java, Indonesia (for *F. gigantica*) or from *Lymnaea tomentosa* snails collected from laboratory snail

Table 1  
Challenge protocol and parasite recovery in sheep given varying challenge doses

Group (n)	Dose (met)	Challenge species	Fluke recovery (mean ± S.E.)	Fluke recovery (range)	% take of metacercariae (mean ± S.E.) <sup>a</sup>	% take (range)
1 (10)	0	–	0		0	0
2 (10)	50	<i>F. gigantica</i>	13 ± 6	4–25	26 ± 13	8–50
3 (10)	125	<i>F. gigantica</i>	29 ± 14	12–52	23 ± 11	10–42
4 (10)	225	<i>F. gigantica</i>	57 ± 20	14–90	26 ± 9	6–40
5 (10)	400	<i>F. gigantica</i>	86 ± 47	5–153	25 ± 14	2–45
6 (9)	250	<i>F. hepatica</i>	119 ± 28	84–166	39 ± 10	27–55

<sup>a</sup> Percentage take is the average percentage of infecting metacercariae recovered as a proportion of counted parasites.

cultures at Elizabeth Macarthur Agricultural Institute, Menangle, NSW Australia (for *F. hepatica*). Sheep were infected with 0–400 metacercariae spread on filter paper and inserted into gelatin capsules (Torpac Inc., Fairfield, NJ, USA) and delivered orally using a dosing gun.

Adult *F. gigantica* and *F. hepatica* parasites were obtained from the livers of infected cattle collected from local abattoirs in Jakarta, Indonesia; and Tongala, Australia, respectively and used to obtain whole worm extracts. Briefly, adult flukes were removed from bile ducts of naturally infected cattle and about 50 flukes were put into 100 ml PBS at 37 °C for 15 min. Initial regurgitant, containing blood, bile and debris, was removed by washing parasites in PBS containing an antibiotic and then live flukes were removed and homogenised in 0.1% Triton X in 90 mM HEPES, 5 mM EDTA (0.05 g parasite/500 µl of buffer) using an Ultra Turrax at 4 °C. Homogenates were rocked on ice at 4 °C for 1 h and then the whole worm extract supernatant was collected after centrifuging at 13,000 rpm (355 × g) for 20 s. The antigen supernatant was stored in small aliquots at –80 °C until required.

The protein concentration of worm homogenates was estimated using a standard assay and reagents from Biorad according to the manufacturer's specification, using Bovine Serum Albumin as standard. All ELISA plates and 24-well tissue culture plates were purchased from Flow Laboratories Inc., USA and Greiner Labortechnik, Austria, respectively.

### 2.3. Experimental treatments

A summary of the design of the experiment is shown in Table 1. Group 1 acted as a control uninfected group, and groups 2–5 received incremental challenge doses of *F. gigantica* metacercariae (50, 125, 225 and 400 metacercariae, respectively). The doses were chosen to represent a linear incremental *F. gigantica* challenge burden on a log scale. Group 6 received a challenge dose of 250 *F. hepatica* metacercariae. The experiment was

conducted over 10 weeks in order to comply with Australian quarantine regulations to dispose of all animals during the pre-patent period of *F. gigantica*.

### 2.4. Haematological and serological measurements

Blood samples were collected in 10 ml EDTA tubes from all sheep before challenge, at time of challenge and 3 days after challenge, and on weekly intervals following challenge. The blood was processed for assessment of total and differential leucocyte counts, haemoglobin level, and packed cell volume (PCV) using a Technicon H1 full haematological analyser.

Sera from blood samples were collected and the level of anti-*Fasciola* antibodies in serum determined by ELISA. Briefly, ELISA plates (NUNC) were coated overnight at 4 °C with 10 µg/ml of whole worm extract antigen of *F. gigantica* or *F. hepatica* (for the detection of anti-*F. gigantica* or anti-*F. hepatica* antibodies, respectively) in 100 µl of carbonate buffer, pH 9.6. ELISA analysis was performed as previously described by Hansen et al. (1999). For the detection of sheep IgG antibodies a 1/1000 dilution of HRP conjugated donkey anti-sheep/goat total IgG (H + L; Silenus) was used. Antibody isotypes were detected with specific non-conjugated mouse anti-sheep IgG<sub>1</sub>, IgG<sub>2</sub> (Beh, 1987, 1988) added at 1:500 and 1:1000 dilution, respectively, or IgA using specific non-conjugated mouse anti-bovine IgA (VRMD) added at 1:1000. Following incubation, plates were incubated with sheep anti-mouse Ig-HRP (1:1000; Amrad Biotech). Sheep endpoint ELISA titres defined as the highest dilution of sera yielding an OD<sub>450</sub> of 0.2, were calculated as described by Windon et al. (2001).

### 2.5. Production traits

Individual non-fasted body weight and group feed-intake were recorded weekly for the duration of the experiment. Feed intake was determined by weighing

daily feed residues after offer of known amounts of food on a daily basis.

### 2.6. Plasma enzyme assays

Liver damage was assessed spectrophotometrically by measurement of plasma levels of the two hepatic enzymes, glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (GGT) on a Cobas Mira chemical analyser using commercial kits (Roche). Levels are expressed as international units per litre (IU/l).

### 2.7. Parasitology

All sheep were euthanised through intravenous injection with 20 ml of Valbarb at 10 weeks post challenge. The sheep were euthanised over a 3-day period (15, 25 and 20 sheep on the first, second and third days, respectively). All livers, bile ducts and gall bladders were collected and examined for the presence of either *F. gigantica* or *F. hepatica* parasites as described (Roberts et al., 1997b). Total parasite number and the parasite biomass based on wet fluke weight were determined from each processed liver. Livers were weighed and subjective scores of macroscopic liver damage (ranging from 0 to 5) were obtained at the time of dissection: where 0 represented no overt signs of tissue necrosis or liver nodules, 1 represented slight liver necrosis or nodules generally confined to less than 5% of the liver, 2 represented light liver damage with liver damage up to 15% of liver surface, 3 moderate liver damage with nodules confined to approximately 30% of the liver surface, 4 represented heavy liver damage with nodules up to 50% of liver surface, and 5 represented extensive liver necrosis with >50% of liver surface showing signs of liver nodules.

### 2.8. Statistical analyses

Data from each biochemical indicator were analysed separately using Genstat. For analysis of the host responses to infection the data was analysed initially by repeated measures analysis of variance to determine if the data needed to be transformed. A split plot general analysis of variance with contrasts was used to account for changes in all parameters between and within groups. Where significant effects were found comparisons were made using least squares differences of the means (LSD, Genstat). This allowed for adjustment of unbalanced data with only nine animals in group 6.

## 3. Results

### 3.1. Parasitology

A strong dose response was evident in the number of parasites recovered, as well as the parasite biomass, in the groups dosed with incremental numbers of *F. gigantica* metacercariae (Fig. 1a and b; Table 1). Across the four experimental groups challenged with *F. gigantica*, parasite recovery ranged from 4 (observed in group 2) to a maximum of 153 flukes (observed in group 5). Mean wet weight per fluke was relatively constant among the three experimental groups challenged with 125, 225 and 400 *F. gigantica* metacercariae at 0.013–0.014 g/fluke (Fig. 1c) with the individual fluke wet weights ranging from 0.003 to 0.023 g/fluke. Despite the increasing challenge dose of *F. gigantica*, % parasite recovery or “take”, defined as the mean percentage of infecting metacercariae recovered as adult flukes, was uniform within the range of 23–26% across the four experimental groups (Fig. 1d, Table 1).

The group challenged with *F. hepatica* showed the greatest number of flukes recovered/sheep (mean 119, range 84–166) (Fig. 1a); a significantly higher biomass of parasites recovered (on average 2.5-fold greater than the highest dose of *F. gigantica*) (mean 2.6 g, range 0.9–4.7 g, Fig. 1b), a higher average fluke wet weight (Fig. 1c; and on average a greater % take at 39.3% (range 27–55%) than any of the four groups challenged with *F. gigantica* (Fig. 1d).

Livers from all control sheep were macroscopically normal (all score 0). Livers from sheep given the highest dose of *F. gigantica* and *F. hepatica* (groups 5 and 6, respectively) showed on average the greatest liver damage with mean lesion scores of 3.0 and 3.3, respectively, and group 6 had significantly higher scores than those of groups 1–4 (Fig. 1e) ( $p < 0.05$ ). No significant increase in liver weight was observed for the sheep challenged with *F. gigantica* compared with the non-infected control sheep. Sheep challenged with *F. hepatica* showed a significant increase in liver weight over control sheep or sheep challenged with *F. gigantica* ( $p < 0.01$ , Fig. 1f). A positive correlation across the groups challenged with *F. gigantica* was evident between liver score, challenge dose and total number of parasites recovered. There appeared to be a positive correlation, at the individual sheep level, between fluke wet weight and fluke burden for *F. gigantica* ( $r^2 = 0.36$ ,  $p < 0.01$  polynomial regression, Fig. 2a) and *F. hepatica* ( $r^2 = 0.47$ ,  $p < 0.04$ , Fig. 2b).

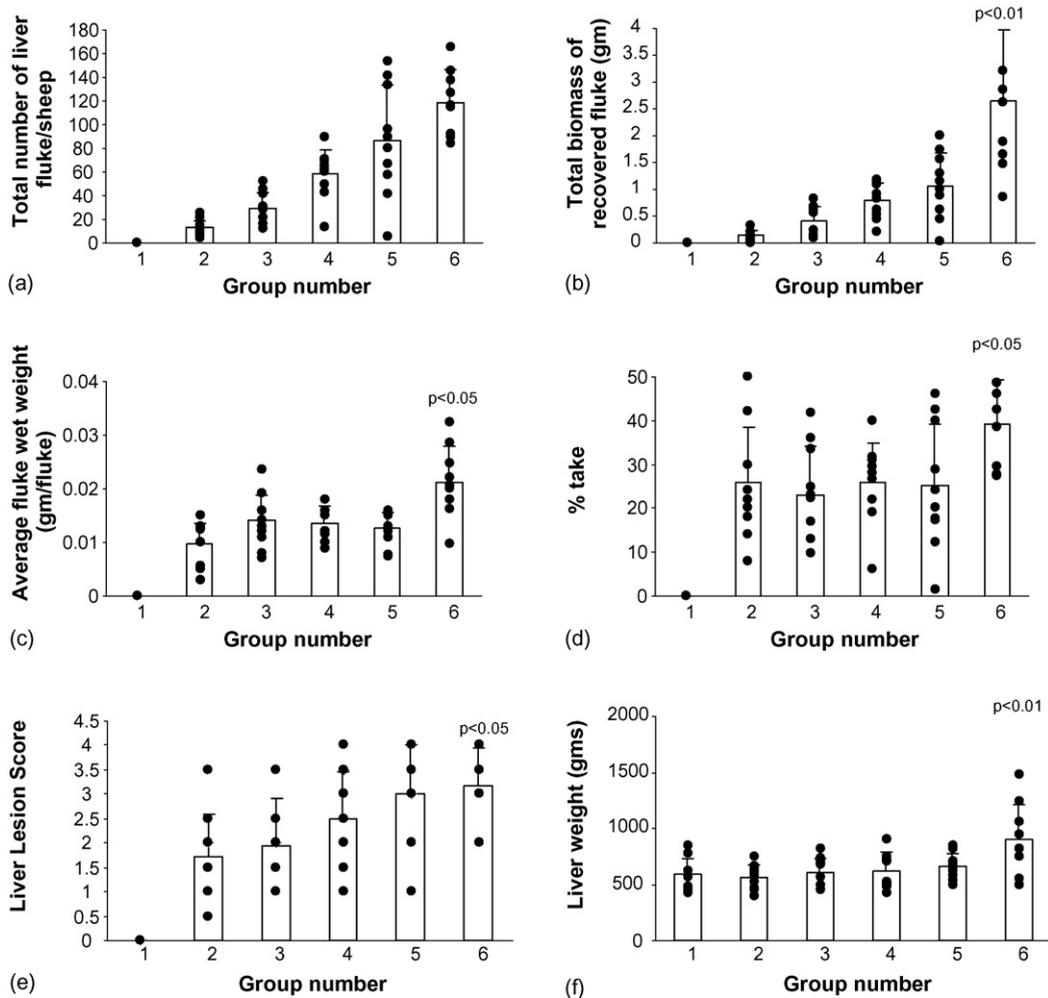


Fig. 1. Parasite recovery and macroscopic liver data from sheep challenged with *F. gigantica* or *F. hepatica*. (a) Liver fluke burdens, (b) total biomass of recovered flukes (g), (c) average wet fluke weight (g/fluke), (d) % take—a measure of the liver fluke recovery as a % of the original challenge dose, (e) macroscopic liver lesion scores, and (f) liver weight (g). Individual points represent individual sheep receiving different doses of *Fasciola* metacercariae. Groups 1–5 were given 0, 50, 125, 225 and 400 *F. gigantica* metacercariae, respectively, and group 6 received 250 *F. hepatica* metacercariae. Columns represent mean and S.D.

### 3.2. Plasma levels of glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (GGT)

The plasma GLDH and GGT levels were measured at day 0 and then at 4, 6, 8 and 10 weeks post infection (Fig. 3a and b). The group challenged with the lowest dose of *F. gigantica* (group 2) showed no detectable increase in GLDH over uninfected controls. By 6 wpi *F. gigantica* infected groups 3–5 had significantly elevated levels of GLDH compared to the uninfected sheep ( $p < 0.005$ ). The *F. hepatica* challenged group had an even higher level (four-fold) of GLDH compared to the *F. gigantica* infected groups at 6 wpi ( $p < 0.001$ ). By 10 wpi the GLDH levels in the group challenged with *F. hepatica* had declined to those seen in the groups

challenged with the highest three doses of *F. gigantica*: at this time point all four *F. gigantica* groups still showed a significantly higher level of GLDH over uninfected control animals. Elevated levels of GGT, as an indicator of epithelial damage in the bile duct, was only seen in the group challenged with *F. hepatica* at 10 wpi when it rose from a mean of <100 IU/l to a mean of 250 IU/l ( $p < 0.0001$ , Fig. 3b) whereas no detectable increase in GGT was observed in any of the groups challenged with *F. gigantica* (Fig. 3b).

### 3.3. Haematology

White blood cell (WBC) counts were measured 1 week prior to challenge, at time of challenge and every

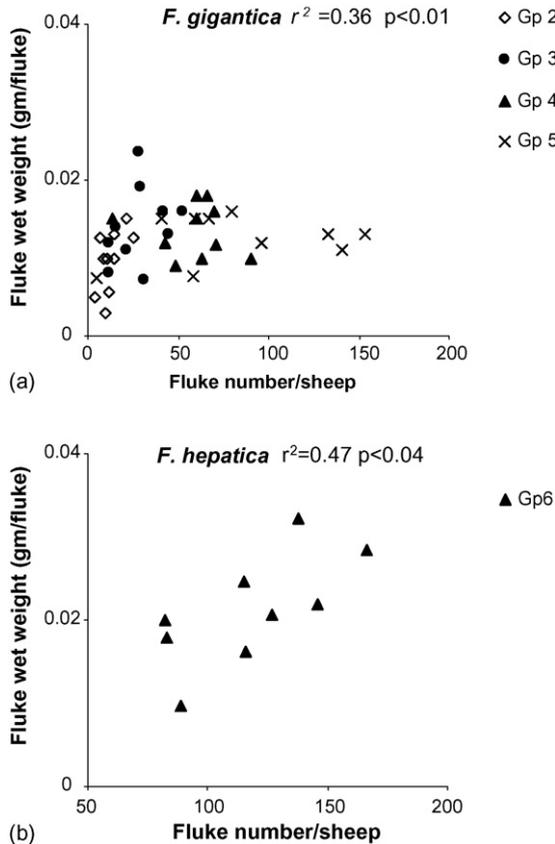


Fig. 2. Relationship between fluke biomass and liver fluke recovery from sheep challenged with *F. gigantica* or *F. hepatica*. (a) Correlation of average fluke wet weight (g/fluke) and total fluke numbers/sheep in sheep challenged with *F. gigantica* (groups 2–5)  $r^2 = 0.36$ ,  $p < 0.01$ . (b) Correlation of average fluke wet weight (g/fluke) and fluke numbers/sheep in sheep challenged with *F. hepatica* (group 6)  $r^2 = 0.47$ ,  $p < 0.04$ .

week until 9 weeks post challenge in all groups (Fig. 4a). At 3 wpi all groups challenged with *F. gigantica* and *F. hepatica* had a mean WBC count significantly greater than the uninfected group ( $p < 0.05$ ). The changes in WBC profile in the group infected with *F. hepatica* appeared to be biphasic with the initial peak at 4 wpi and a further substantial increase in the WBC count at 9 wpi ( $p < 0.001$ ). This biphasic response was not evident in the groups challenged with *F. gigantica*. In the final 2 weeks of the experimental period the group challenged with the lowest dose of *F. gigantica* had a WBC concentration which was no different from uninfected control groups. The group challenged with *F. hepatica* on the other hand reached a peak WBC response at week 9 immediately prior to slaughter (Fig. 4a).

The levels of haemoglobin (HGB) (g/L) generally increased steadily for all groups over the first 8 weeks post infection (Fig. 4b). The uninfected group of sheep

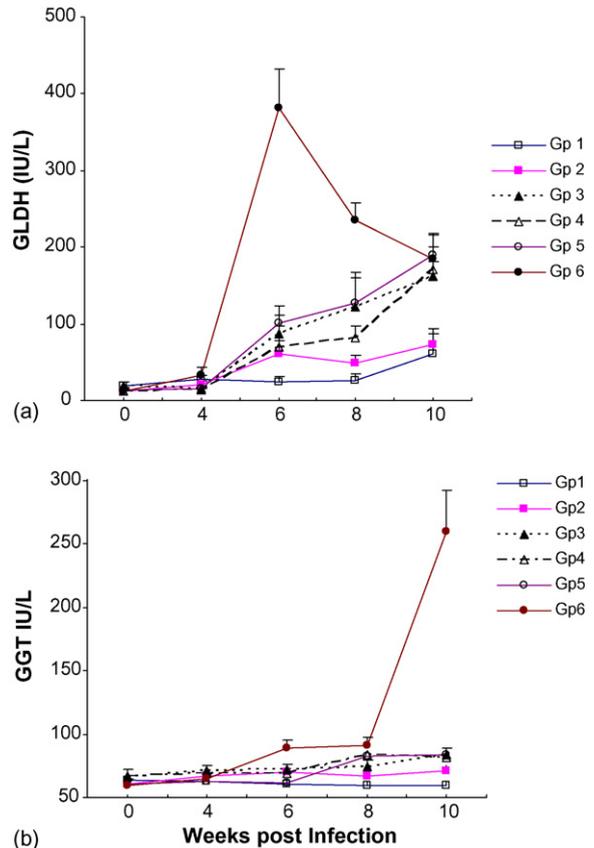


Fig. 3. Plasma glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (GGT) levels (mean  $\pm$  S.E.M., IU/l).

showed no significant difference to any of the groups infected with *F. gigantica*. By 9 wpi there is a significant fall in HGB level in the group infected with *F. hepatica* compared to all other groups ( $p < 0.001$ ). The packed cell volume (PCV%) in all groups mirrors the trend seen in the HGB profiles. PCV gradually increased in all groups and at 9 wpi there was a sharp decrease in the *F. hepatica* infected group relative to the rest of the groups ( $p < 0.001$ , Fig. 4c).

An initial rise of mean circulating eosinophil numbers occurred at 2 wpi in all infected groups. At 3 wpi the mean eosinophil numbers increased from basal levels of less than  $3 \times 10^9$  up to  $15 \times 10^9 \text{ l}^{-1}$  in the groups infected with *F. gigantica* ( $p < 0.001$ ) and to almost  $25 \times 10^9 \text{ l}^{-1}$  ( $p < 0.0001$ ) in the *F. hepatica* challenged group (Fig. 5a). Relative to the control group, the mean eosinophil counts in groups 2–5 remained elevated throughout the entire experimental period post infection until time of slaughter. A strong biphasic response in eosinophil count was observed in the group challenged with *F. hepatica*, peaking for the first time at 4 wpi ( $27 \times 10^9 \text{ l}^{-1}$ ) followed by a decline to  $14 \times 10^9 \text{ l}^{-1}$  at

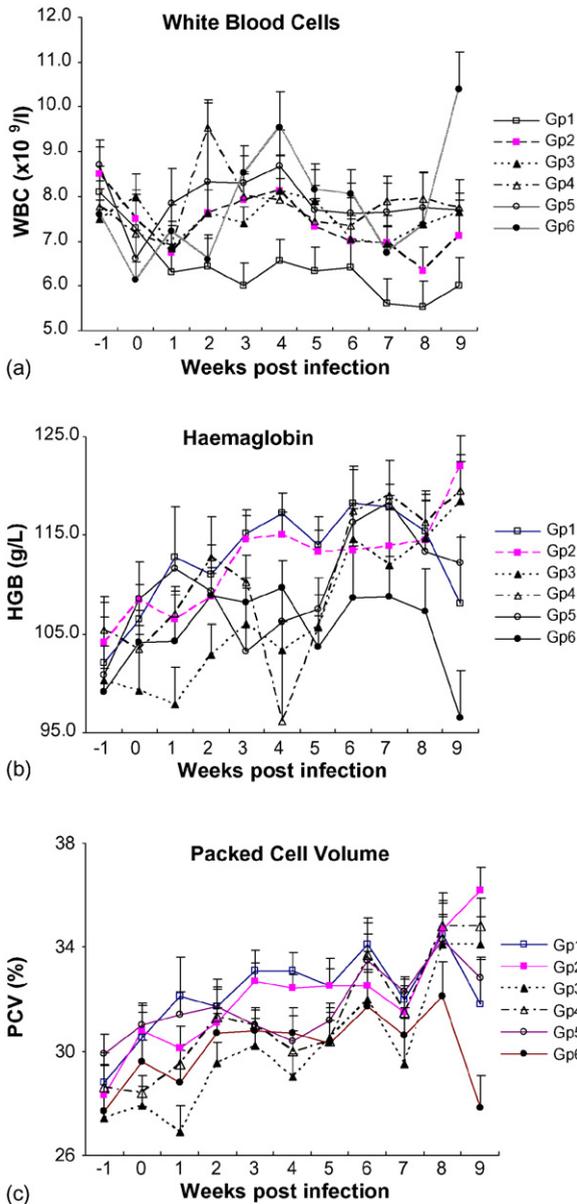


Fig. 4. Longitudinal profiles of infection parameters throughout the study period. (a) White blood cell counts (WBC) ( $\times 10^9$  cells/l); (b) haemoglobin (HGB) (g/l); (c) packed cell volume (%). Error bars indicate the standard error of the mean where  $n = 10$  for groups 1–5 and  $n = 9$  for group 6.

6 wpi and followed by a steady increase to a mean  $30 \times 10^9 l^{-1}$  at 9 wpi. This biphasic response was not evident in any of the groups challenged with *F. gigantica*. When the eosinophil count of the combined groups of *F. gigantica* infected animals are compared to the single *F. hepatica* challenged group of animals at 9 wpi there is a clear indication that *F. hepatica* appears to induce a greater eosinophilia ( $p < 0.01$ ).

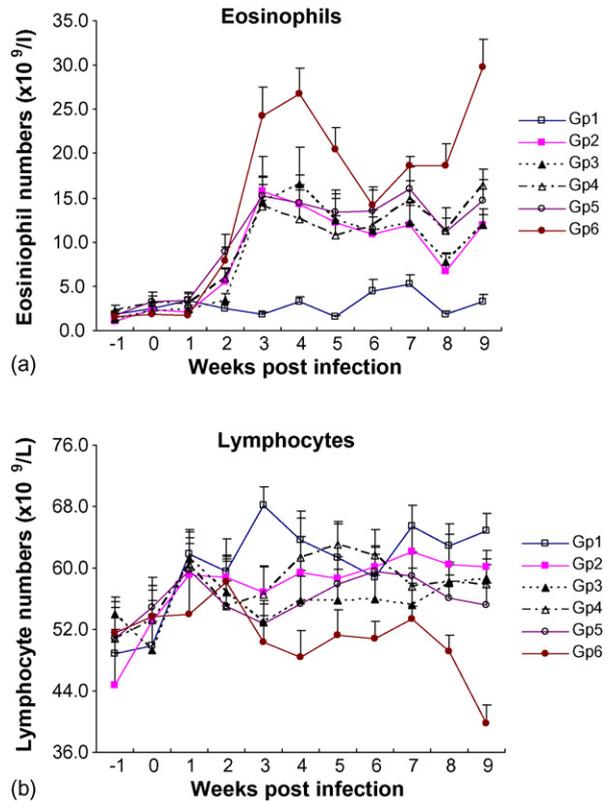


Fig. 5. Longitudinal profiles of infection parameters throughout the study period. (a) Eosinophil counts ( $\times 10^9$  cells/l) and (b) lymphocyte counts ( $\times 10^9$  cells/l). Error bars indicate the standard error of the mean where  $n = 10$  for groups 1–5 and  $n = 9$  for group 6.

There was no significant difference in lymphocyte counts between groups until 8 wpi when the group challenged with *F. hepatica* fell significantly below control levels ( $p < 0.05$ ) and continued to fall by 9 wpi ( $p < 0.001$ , Fig. 5b). Compared to the control group, there was a steady decrease in neutrophil numbers in all infected groups until 3 wpi when all groups showed a plateau (data not shown). There was no significant difference in basophil or monocyte numbers between any of the groups across the experimental period (data not shown).

### 3.4. Specific serum immunoglobulin responses to whole worm extract during infection with *F. gigantica* and *F. hepatica*

At 1 wpi all groups infected with either *F. gigantica* or *F. hepatica* showed an elevation in anti-*Fasciola* IgA titres which remained high until week 5 when they began to decline to control levels (Fig. 6a). There was very little difference in anti-*Fasciola* specific IgM levels in all groups throughout the period of measurement



significantly different from the uninfected control group at 5 and 7 wpi. The ratio of IgG<sub>1</sub> to IgG<sub>2</sub> showed no clear responses consistent with the challenge dose or species of *Fasciola* (Fig. 6e).

Measurement of IgE in all samples was attempted: however, unlike the other isotypes which were measured within 2 months of sampling there was a delay in the analysis and IgE levels were low or undetectable following long term storage and transportation, possibly due to degradation of the samples.

### 3.5. Body weight and feed intake

The cumulative changes in body weight over the duration of the experiment are shown in Fig. 7a. All animals gained weight during the entire experimental period as a consequence of being on *ad libitum* food intake and at a young growing age. However, when comparative relative growth rates in infected animals were compared with non-challenged control animals, significant differences were detected between parasite infected groups (Fig. 7b). The body weights taken every 3 days prior to challenge showed very little change in all groups. In the first wpi the body weights of all sheep decreased by 1.5 kg on average. The animals in the

groups challenged at the two highest doses of *F. gigantica* (groups 4 and 5) and the group challenged with *F. hepatica* (group 6) showed a similar and significant decrease in bodyweight relative to uninfected controls by 2 wpi and were not able to recover by way of compensatory growth for the remainder of the experimental period. Group 4 sheep (mean 57 flukes) were infected with about 50% of the parasite burden observed in group 6 (119 flukes), suggesting that, above a parasite threshold of about 57 *F. gigantica* parasites, the loss in body weight is similar even though the mean biomass of *F. gigantica* flukes in group 4 is about three-fold lower than that seen with *F. hepatica* in group 6 (Fig. 1b). The two groups challenged at the lower doses of *F. gigantica* tended to retain growth rates above or near those seen in the control group, indicating that at these levels of infection (mean 13–29 flukes) the animal's body weight is uncompromised. Feed intake measurements were not started until week 1 post challenge. There was no significant difference between groups in feed intake during the post challenge period indicating no decrease in appetite (data not shown). Relative to groups 1–3, the reduced growth rate in sheep challenged with *F. hepatica*, and two groups challenged with the highest dose of *F. gigantica*, may therefore indicate a real reduction in energy expenditure attributable to disease and fasciolosis.

## 4. Discussion

There have been few studies that directly compare the pathogenicity of *F. gigantica* and *F. hepatica* (Boyce et al., 1987; Spithill et al., 1999; Piedrafita et al., 2004; Zhang et al., 2004, 2005a,b). Earlier studies have suggested that *F. gigantica* is more pathogenic than *F. hepatica* based on the finding that fewer parasites killed sheep and the larger size of *F. hepatica* flukes in mature infections (Roberts et al., 1997a). However, these studies measured comparative pathogenicity after long term infections with mature parasites. In addition, many of the early studies on these two parasite species assumed that infection with *F. gigantica* was essentially similar to infection with *F. hepatica*, since the two parasites infect the liver and have a similar life cycle: this has led to the assumption that they are essentially similar organisms epidemiologically and in terms of the host-parasite relationship (Spithill et al., 1999). However, *F. gigantica* is slower to develop in ruminant hosts and takes greater than 14 weeks following infection to reach the bile ducts while *F. hepatica* parasites establish within the bile ducts after just 8–10 weeks of infection (Wiedosari and Copeman, 1990; Behm and Sangster,

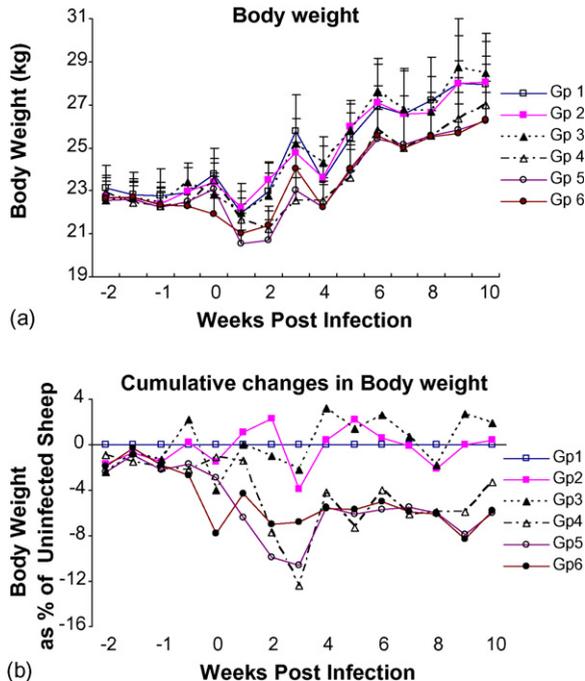


Fig. 7. Longitudinal profiles of production parameters in different groups of sheep challenged with *F. gigantica* or *F. hepatica*. (a) Cumulative body weights and (b) changes in body weight as a % of control sheep. Error bars indicate the standard error of the mean where  $n = 10$  for groups 1–5 and  $n = 9$  for group 6.

1999). Just prior to the migration of *F. hepatica* into the bile ducts, a rapid growth of the parasite occurs during liver migration from 5 to 8 weeks post-infection (Dawes and Hughes, 1964). We wondered whether this putative earlier development and maturation of *F. hepatica* in sheep would cause more host damage early in infection when compared to *F. gigantica* infections. We, therefore, conducted a detailed comparative trial in the susceptible Merino breed for the first 10 weeks post infection as this is the pre-patent period of *F. gigantica*, and would allow the evaluation of the early comparative host immune responses to both *F. gigantica* and *F. hepatica* infection.

Previous limited studies have shown that when an equal number of *F. hepatica* or *F. gigantica* metacercariae are administered to ruminants, more flukes are recovered from the *F. hepatica* infected ruminants than the *F. gigantica* infected ruminants (Zhang et al., 2004, 2005a,b). In order to compare the pathogenicity in early infection between the fluke species we, therefore, attempted to attain similar worm burdens between the two groups by performing an incremental response challenge with increasing *F. gigantica* metacercariae in groups of Merino sheep. In this study fluke recovery was similar to the recent reports from Zhang et al. (2005a,b) in that more flukes were recovered from sheep infected with *F. hepatica* (39%) than were recovered from *F. gigantica* infected sheep (26%, group 4) receiving similar numbers of metacercariae. Within the groups dosed with *F. gigantica* a strong dose-dependent response was observed in both fluke recovery and fluke biomass with increasing dose of metacercariae. Interestingly the % take between all the *F. gigantica* infected groups was very even, suggesting a uniform viability of parasite establishment independent of infection dose. The dose of challenge or fluke recovery in *F. gigantica* infected sheep also showed no significant impact on average fluke size, suggesting that there is no obvious sign of competition between flukes for host resources within the first 10 weeks of infection (Fig. 1c).

Based on fluke biomass, average fluke size, degree of liver damage, plasma GLDH levels and GGT responses, this experiment suggests that *F. hepatica* is more pathogenic than *F. gigantica* in the first 10 weeks of infection. This is evident from comparing data in groups 5 and 6 where similar worm burden recoveries were obtained. Within the first 10 weeks of infection, *F. hepatica* develops more rapidly than *F. gigantica* resulting in increased plasma levels of GLDH, indicating greater damage to the liver parenchyma (Boyd, 1962; Meeusen et al., 1995). The earlier maturation of *F. hepatica* was also demonstrated by a

decrease in GLDH in this group of sheep at 8–10 wpi, corresponding to the migration of *F. hepatica* into the bile ducts, and a corresponding increase in the GGT levels at 10 wpi, indicating epithelial damage in the bile duct (Chauvin et al., 1995). In contrast, in the sheep infected with *F. gigantica*, the plasma levels of GLDH were beginning to rise at 6 wpi and continued to rise steadily but did not reach the same peak as the *F. hepatica* infected animals; GGT levels were unaffected by 10 wpi in the *F. gigantica* groups. A slower maturation of *F. gigantica* is also indicated by the finding of smaller, immature flukes in the liver parenchyma at slaughter at 10 wpi compared to *F. hepatica*, where most flukes were observed within the bile ducts. The greater damage to the *F. hepatica*-infected Merino sheep was also evident by the subjective scoring of greater lesions in the *F. hepatica*-infected livers and the compensatory hypertrophy of the liver leading to a significant increase in the relative liver weights. The greater pathogenicity of *F. hepatica* was also evident from the significant decreases in haemoglobin levels and packed cell volume late in infection (9 wpi) compared with all the *F. gigantica* infected groups. It is known that *F. gigantica* remains in the liver for 12–14 wpi and only then migrates to the bile ducts (Spithill et al., 1999).

It has been well documented that infection of some breeds of sheep with *F. hepatica* causes a large infiltration of white blood cells into the liver (Meeusen et al., 1995; Tliba et al., 2000). Here, the white blood cell response and eosinophilia following *F. hepatica* infection was biphasic with the initial peak at 4 wpi and a second peak at 9 wpi. The eosinophilia reported in this study is similar to that documented by others in sheep infected with *F. hepatica* (Chauvin et al., 1995; Zhang et al., 2005b) or *F. gigantica* (Roberts et al., 1997a; Hansen et al., 1999; Zhang et al., 2005b). This biphasic response corresponds to the migratory phase of the juvenile fluke in the liver (week 4) followed by a reduction in peripheral cell counts as the parasite matures in the bile duct (Rushton and Murray, 1977), a finding confirmed by the elevated levels of GGT at 10 wpi in the *F. hepatica* infected group 6, indicating evidence of epithelial damage in the bile duct. When the eosinophil numbers in the groups with incrementally increasing doses of *F. gigantica* were analysed there was no significant difference between the groups, indicating that added worm burden had no additional effect on the eosinophilia.

Pooled sera of each group were used to measure the specific humoral immune response to whole worm extracts to attain general trends in the antibody

responses following *Fasciola* infection. Specific IgG<sub>1</sub> was produced in all infected animals as early as 1–3 wpi and maintained at high levels throughout the 10 weeks of infection. This response was similar in all sheep infected with varying doses of *F. gigantica*, and for the *F. hepatica* infected group, as previously observed by others (Santiago and Hillyer, 1988; Chauvin et al., 1995; Moreau et al., 1998; Zhang et al., 2004). The changes in IgA response were less defined with a small peak around 5 wpi, but otherwise no trend was observed which could be clearly attributed to infection dose. With IgG<sub>2</sub> there was a trend toward higher titres in sheep infected with the lower worm burdens (groups 2–4), suggesting that higher *F. gigantica* and *F. hepatica* burdens suppress IgG<sub>2</sub> responses in Merino sheep. Suppression of IgG<sub>2</sub> responses to *F. gigantica* infection was observed in Indonesian Thin Tail sheep, relative to Merino sheep (Hansen et al., 1999). Moreau et al. (1998) have previously shown that the IgG<sub>2</sub> response to a cathepsin L vaccine correlates with reduced worm burdens in vaccinated cattle, implying a protective role for this isotype against *F. hepatica*. These observations raise the interesting prospect that *Fasciola* parasites produce a factor (s) that can suppress IgG<sub>2</sub> responses. In ruminants, the IgG<sub>2</sub> response is regulated by IFN-gamma (Estes et al., 1994).

Very few studies have been able to successfully compare the impact of *F. gigantica* and *F. hepatica* parasitism on production, feed intake and host responses. This study shows that the severity of infection by both *Fasciola* species directly influences weight gain within 2 wpi. In this experiment the level of nutrition was not restricted, as is normally seen under production systems, and therefore the impact on production may be expected to be of even greater consequence under field conditions. High doses of both *F. gigantica* and *F. hepatica* were able to induce similar effects on production, despite the greater damage caused by *F. hepatica* infection based on fluke biomass and liver damage. This suggests that production losses are not solely related to fluke biomass and liver pathology; rather, there is a parasite threshold above which production losses are comparable with *F. gigantica* and *F. hepatica*.

In conclusion, the development and subsequent effect on sheep of the two *Fasciola* species is quite different. During the early stage of infection in a permissive host, *F. hepatica* appears to be more pathogenic than *F. gigantica* due to its faster rate of growth and the speed of its progression through the migratory phases to establishment in the bile duct. Relative to *F. gigantica*, *F. hepatica* appears to express a

greater capacity to induce eosinophilia, and an IL5-like activity has been described in ES of *F. hepatica* (Milbourne and Howell, 1993). Moreover, studies in Indonesian Thin Tail sheep have shown that sheep can acquire resistance to infection by *F. gigantica* but not *F. hepatica*, suggesting that subtle biochemical differences exist between these two parasite species (Roberts et al., 1997a,b; Piedrafita et al., 2004). Further comparative studies on these two *Fasciola* species are needed to define the basis for these differences in their parasite-host interactions which may identify parasite mechanisms that could be targeted for control of fasciolosis.

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