Phenotypic analysis of host-parasite interactions in lambs infected with Teladorsagia circumcincta

Phenotypic Analysis of Host-Parasite Interaction in Lambs Infected with *Teladorsagia circumcincta*

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Abstract

Teladorsagia circumcincta has a widespread deleterious impact on sheep welfare and economic production. In order to devise effective methods of integrated control and selective breeding, it is necessary to understand the interaction between sheep and *T. circumcincta*. In this study, female Blackface lambs expected to be genetically variable for resistance to gastrointestinal nematodes were either exposed to a continuous experimental infection of *T. circumcincta* or sham dosed to monitor the phenotypic response to infection. As a measure of parasitism and host response, faecal eggs were counted over a three month period and post-mortem burdens ascertained. The host response to the infection was also described by differential count of white blood cells, IgA antibody level, and variation in body weight. Results suggest that nematode abundance (mean number of parasites per host) and prevalence (proportion of infected animals) were maximal shortly after the beginning of infection when virtually all the flock was infected and shed worm eggs. The host response was associated with increasing IgA antibody levels and eosinophil concentrations which, the data suggest, caused a reduction of total adult worms and an increase in the frequency of EAL4 (early arrested L4) worms.

1. Introduction

The sheep farming industry depends on economically viable meat and wool production systems for its sustainability. Gastrointestinal nematodes are an important factor in production losses (Brunsdon, 1988) and with the occurrence of anthelmintic resistance to broad spectrum drugs (Coles et al., 2006), the search for additional methods of parasite control has never been more necessary (Eady et al., 2003). In order to delay the rate of evolution of anthelmintic resistance, new recommendations for integrated control include the exploitation of genetic variation for resistance found within and between breeds, and efforts have concentrated on identifying genes associated with parasite resistance (Dominik, 2005).

Blackface sheep are the most numerous breed in the United Kingdom (National Sheep Association, 1998) and the most abundant parasite they encounter is the nematode *Teladorsagia circumcincta* (Stear et al., 2007). Much work has focussed on the phenotypic manifestation and genetic control of resistance to *T. circumcincta* in this breed (Stear et al., 1997). These studies typically involve natural infections of mixed helminth species or anthelmintic bolus experiments in lambs previously exposed to nematodes, and often involve periodic anthelmintic treatment (Stear et al., 1995; Henderson and Stear, 2006). Confounding effects due to previous infections, treatments, or records collected at distant time points (months, years) make it difficult to work out what mechanisms underlie the interaction between host and parasite. This information would be particularly useful for determining sustainable parasite control strategies in situations where the use of anthelmintics is limited or not possible.

In this study naïve lambs with diversity in their predicted genetic resistance were trickle infected with *T. circumcincta* and compared with control lambs. The objectives were first, to characterize the worm population, second to track the course of infection, and third, to determine whether predicted breeding values for faecal egg count (FEC) were associated with phenotypic variation.

During the experimental period we found the severity of infection (measured as FEC) to be highest shortly after the beginning of the infection and lambs' resistance was associated with increased high levels of IgA anti-*T. circumcincta*. The adult worms were highly aggregated in relatively few lambs and virtually absent in the more resistant lambs, while the early arrested larvae (EAL4) were uniformly distributed across the flock. Finally, the genetic merit for FEC (estimated breeding values) was associated with several traits including IgA responsiveness and body weight.

2. Materials and method

2.1 Animals and experimental design

The study animals were 57 female Blackface lambs bred at the Roslin Institute (Edinburgh, UK). This study focused on female lambs as it is in this sex and age class that the strongest immune response can be observed (Smith et al., 1985; Gulland and Fox, 1992; Barger, 1993). An additional criterion for choosing the study animals was that each of the 57 lambs had a twin so that the variation due to rearing conditions was reduced. The parents used to generate the study lambs belonged to a Blackface sheep population previously used for quantitative genetic and QTL analyses of faecal egg count (Davies et al., 2006) and estimated breeding values for FEC of the parents were available. The dams were drawn from a normal distribution of lamb FEC breeding values, divided into those above and below the mean. The mean breeding value for log-transformed

FEC for the top-half dams was 0.193 (SD= 0.160, n= 23) while for the bottom-half the mean was 0.573 (SD= 0.138, n=19). The three sires had intermediate breeding values (0.468, 0.228, 0.063). In order to spread genetic variation in parasite resistance across the progeny, each sire was mated with an equal number of dams from the top-half and bottom-half of the breeding value distribution. Hereafter, lambs born from dams belonging to the top-half and bottom-half of the breeding value distribution will be referred to as the resistant breeding value group and

susceptible breeding value group, respectively. All the lambs were born between the 30th March and the 24th April and were weaned at the age of approximately three months.

The lambs were as free of parasite infection as possible at the start of the experiment. Pregnant ewes were brought indoors on the 1st February and newborn lambs remained indoors throughout the experimental period. When brought indoors, the ewes were treated with three different parasiticides (moxidectin (Cydectin, 1ml/5kg body weight), levamisole (Levacide, 0.5ml/2kg), fenbendazole (Panacur, 2.5-3ml/sheep)) and regular faecal samples were collected to check for the presence of infections. Although faecal samples were always negative for strongyle eggs in ewes and in lambs before experimental infection, a moderate coccidia infection was observed in some ewes and high coccidia counts were found in almost all lambs. Very low *Strongyloides papillosus* egg counts were also detected in a few lambs. Ewes and lambs were bedded on clean straw and fed *ad libitum* with Excel Ewe nuts (18.0% protein, 13% metabolizable energy; Carrs Billington, Carlisle, UK) and Maize Lamb Pellets (16.0% protein, 13% metabolizable energy; Carrs Billington, Carlisle, UK). The welfare of the animals was continually monitored and veterinary treatments were deemed necessary during the course of the experiment, especially for coccidiosis and respiratory infection (Table S1).

Ten of the 57 lambs were chosen as controls and kept uninfected. The two breeding value groups were equally represented in the control flock (five resistant and five susceptible lambs) and each control lamb had a twin in the infected set. Apart from these criteria the choice of the control lambs was random. The remaining 47 lambs (25 resistant and 22 susceptible lambs), maintained in the same barn as the controls but in a separate pen, were experimentally infected with approximately 2,300 infective larvae three times a week starting on the 10th of July 2006 and continuing until sacrifice. Control lambs were sham dosed with tap water at the same time. The infected animals included five pairs of twins and, as stated also above, each control lamb had a twin in the infected group.

2.2 Data collection

Faecal and blood samples were collected on alternate weeks starting from the 21st June for FEC (19 days before experimental infection), the 10th of July for blood samples (0 days post infection, dpi), and the 12th July (2 dpi) for body weight. All the lambs were sacrificed between the 12th and 18th October (94-100 dpi). The calendar of sampling is represented in Figure 1.

2.3 Measures of parasitism: faecal egg count (FEC) and post-mortem worms

Faecal samples were taken directly from lambs on the same day or within the same week at a standardised time of day. FEC for *T. circumcincta* was determined as the number of parasite eggs per gram (wet weight) of faeces using a modification of the McMaster technique which detected a minimum of 50 eggs per gram (MAFF, 1986). To improve the likelihood of detection, two replicate counts (that is two separate McMaster counts) were performed so that at least 25 eggs per gram could be detected. Inadvertent infections of *Strongyloides papillosus* and *Eimeria* spp were similarly quantified (data not shown). The examination of the *post-mortem* worm burden in the abomasum (see below) and intestine (not shown) did not detect any nematode species other than *T. circumcincta*

and *Strongyloides papillosus.* Since eggs from this latter species are easily distinguishable from strongyle eggs, there was reason to believe that the strongyle FEC presented here relates exclusively to *T. circumcincta* eggs.

T. circumcincta individuals were recovered for direct quantification by collecting the contents of the abomasum and rinsing the mucosal lining with water into a receptacle to a volume of 10L. Following thorough mixing, a 10% aliquot was removed and passed through a 38 mm sieve. The saved material was stored in 5% formalin solution for later quantification (see below). Half of the abomasum wall was used for parasitology sampling, the mucosa peeled from the wall and frozen at -20oC. It was later subjected to peptic digestion for the recovery and enumeration of arrested larvae (MAFF, 1986). *T. circumcincta* counts were made on a

2% aliquot (or 5% where total count was very low). The developmental stages were categorised on the basis of morphology as 4th stage early arrested larvae (EAL4), 4th stage larvae (L4), 5th stage larvae (L5, unsexed immature adults), male and female adults. Small intestine contents from all lambs were sampled in the same way to enumerate the adult *S. papillosus* population (data not shown).

Up to 20 female worms per infected sheep were stored in 10% formalin solution. *In utero* eggs were counted by microscope after iodine staining. The length of the worms was then measured on digital microscope images. It has been found that female worms are slightly longer than the males and sheep with longer females also have longer males. Therefore, since female length can be correlated with *in utero* eggs, only female worms were measured (Stear et al., 2002).

2.4 Blood samples

Sampling and processing

Blood was taken by jugular venipuncture using K3-EDTA vacutainer tubes (Becton Dickinson, Oxford, UK) at fortnightly intervals at a standardised time of day. The blood processing described below began within a few hours after sample collection and was completed within 48 hours.

Total white blood cell counts

Manual counts of white blood cells were performed on a Neubauer improved bright-line haemacytometer (Assistent®, Germany) according to manufacturer's instructions. Blood was prepared by inverting the vacutainer tube eight times before removing 0.1 ml of blood and mixing this with 0.9 ml diluting fluid (10 ml glacial acetic acid + 4 ml ethanol + 180 ml

distilled water + a small drop of methylene blue dye). The mixture was left for 10 minutes, mixed and then loaded onto the slide.

Differential white blood cell counts

A feather edged blood smear was prepared by spreading a dot of mixed blood from a capillary tube along a slide and allowing to air dry (Weiser, 1981). The slide was stained using Wright-Giemsa stain for 30 seconds, soaked in distilled water for 10 minutes then rinsed and allowed to dry. The slide was mounted and read under oil at 1000x magnification using the battlement method (Thrall and Weiser, 1997). At least 100 white blood cells were differentiated into lymphocyte, neutrophil, eosinophil, monocyte and basophil and absolute counts of each obtained by relating back to the haemacytometer counts.

2.5 Preparation of L3 antigen and IgA antibody assay

Whole *T. circumcincta* L3 larvae were collected from a monospecific culture passaged using the method described by Seaton et al. 1989. These were centrifuged and resuspended in PBS at approximately 500µl pellet/1ml PBS and disrupted using a ribolyser (Ribolyser, Hybaid, www.thermohybaid.com) for 1 minute, cooled on ice, repeated until the worms were entirely disrupted as seen under microscope. The resulting suspension was then centrifuged and the supernatant collected, and repeated by resuspending in PBS until the supernatant ran clear. Protein in the supernatant was measured using Bicinchoninic acid method (Sigma, Poole, UK) and solution adjusted so that the 1:1000 dilution corresponded to 1µg/ml.

After white cell aliquots were taken off, the blood sample was centrifuged (10 minutes at 3000g) and plasma taken off and frozen at -20^{o} C. These aliquots were used for IgA analysis. The amount of IgA anti-*T. circumcincta* in serum was measured by antibody capture ELISA. Flat-bottomed microtitre plates (Greiner High binding ELISA plates, cat. 655061) were

coated with 50 µl of *T. circumcincta* L3 water soluble extract diluted 1:1000 in 0.1M carbonate buffer at pH 9.5 and incubated overnight at 4oC. After washing three times with wash buffer (PBS, 0.05% v/v Tween-20), plates were blocked with 200 µl of 10% dried milk powder (Marvel, Cadbury Schweppes) in wash buffer, incubated at 37^{o}C for half an hour, and then washed again three times with wash buffer. On each plate, a standard serum diluted eight times from 1/10 to 1/1280 was loaded in duplicates (50 µl) along with the sample sera also in duplicates (50 µl diluted 1/20 in wash buffer) and blank controls. After re-washing, 50 µl of mouse anti-bovine/ovine IgA antibody (Serotec, MCA628) diluted 1/1000 in wash buffer were added and incubated for one hour at room temperature, and re-washed with wash buffer. 50ul of Dako rabbit anti-mouse HRP were added to all wells and incubated for one hour at room temperature, and re-washed with wash buffer. Finally, 100 µl of Sigma-fast OPD (Sigma, Poole, UK, made up as per manufacturer instructions) were added to each well, incubated for 5-10 minutes in darkness, and then stopped with 3M HCl. IgA measurements (optical densities read by a spectrophotometer at 492 nm) here reported are concentration relative to the standard serum calculated against the standard curve of each plate (Figure S1). The average coefficient of determination (R^2) of the linear regression fitted through the standard points was 0.968 (min= 0.924, max= 0.989, n= 12 plates). The mean coefficient of variation (CV= (SD/mean) x 100) of the replicates of the standard was 3.34%; the mean CV of the eight dilutions across plates was 10.87%; the mean CV of the sample replicates was 2.95%.

2.6 Data analysis

The two separate McMaster counts (hence based on four chambers) of each strongyle FEC sample were combined into an arithmetic mean. Infection in the experimental flock was quantified as prevalence, i.e. proportion of animals having worms after *post mortem* count,

and as intensity, i.e. mean worm burden of the animals having worms. Prevalence and intensity of FEC was defined similarly. Traits with repeated measures were described by generalized linear mixed models fitting the lamb identity as random effect to account for the temporal pseudo-replication. For the temporal analyses we present the response variables as a function of the days post infection. However, traits were modelled using the animal age as a time variable rather than the days post infection, in order to correct for the slight differences in age between lambs. An overdispersed Poisson distribution, analogous to the negative binomial distribution, was fitted on count data (FEC, worm count) where the explanatory variables were linked to the response by the log function. IgA and blood cell concentrations, excluding WBC, were assumed to follow a gamma distribution with inverse link function. A normal distribution was a good fit for all the other traits (WBC, lamb weight, worm length). Fixed effects such as breeding value group, control/infected status, age, and other phenotypic traits were fitted if they significantly improved the model. Confidence intervals for FEC and *post mortem* data were calculated by bootstrap sampling. All data analyses were performed in R 2.5.1 (R Development Core Team), and the package lme4 (Bates, 2007) was used for fitting general linear mixed models.

3. Results

3.1 Post mortem worm burden and worm characteristics

At the time of sacrifice (12th-18th October), i.e. three months after the beginning of infection, most of the parasites were adults (72.5%) with a significant excess of females (adult sex ratio 55:45 in favour of females, p=0.002 after paired Wilcox test, Figure 2A). On average each infected lamb carried 2700 adult worms but 10 lambs did not have any detectable adult worms (Table 1 and Figure 2B). As shown in Figure 2C, lambs with heavier adult worm burden produced more worm eggs. The Spearman's rank correlation between adult worm count and last FEC was rs= 0.83, p<0.0001 and a linear fit of FEC on adult worm count explained approximately 60% of the variation in *post mortem* adult burden (r2= 0.593).

The larval stages, EAL4, L4, and L5, were less abundant than the adults, on average representing 27.5% of the worm population. In particular, the L5 stage was significantly rarer than the other two larval stages, EAL4 and L4 (p<0.001, Table 1). However in terms of prevalence, i.e. proportion of infected hosts, the larval stages were at least as widespread as the adults. The EAL4 stage, in particular, was found in all but one lamb (98% prevalence, confidence interval between 94 and 100%, Table 1). Indeed, a general linear model revealed that the number of adult worms counted in each sheep was positively correlated with the number of L4 (r= 0.35, p<0.05, df= 45) and L5 larvae (r= 0.54, p<0.001, df= 45) but not with the EAL4 stage. To further characterize the worm population, we compared the worm demography in lambs where eggs could be counted on the last FEC sampling (FEC>0, see below for analysis of FEC) with that in lambs where eggs were below the level of detectability (FEC=0) on the last FEC sampling (Figure 3). Lambs with egg output (FEC>0) carried 3600±470 adult worms and 370±40 EAL4 worms (t-test for different means: p<0.0001, df=26) while lambs with no eggs (FEC=0) carried 310±130 adult worms and

240±60 EAL4 worms (NS, df=27; Figure 3A and B). In terms of frequency, adult worms in the FEC>0 group represented 69±4% of the population but in the FEC=0 group only 21±6% ($p<0.01$, df=26). In contrast, the EAL4 stage was 12±2% in the FEC>0 group but 52±7% in the FEC=0 group ($p<0.01$, df=27; Figure 3B).

On average, each worm had 20 eggs and measured 8.4 mm (Table 2). The variation in length was relatively small, the coefficient of variation of the whole worm dataset being 12.5% (n=632). In comparison to worm length, *in utero* egg count appeared to be more variable with a coefficient of variation of 57%. Across sheep, mean *in utero* egg count was positively correlated with worm length ($r= 0.76$, $p<0.001$, df= 33, Table 2 and Figure 2D) and with the weight of the host ($r= 0.30$, $p<0.05$, df= 35). On average an increment of 1 mm in worm length resulted in 7.8±1.2 more eggs. Individual sheep had partial control over worm fecundity and worm length, since part of the variation in these traits was explained by sheep identity (25% and 22% respectively). Neither worm length nor egg count showed a significant correlation with the total number of worms or with FEC. However, a moderate, positive correlation (r=0.35) was found between egg count and number of female worms ($p=0.03$, df= 35).

3.2 Longitudinal analysis of infection related traits

Several traits likely to be related to response to parasites were measured during the course of infection in both experimental and control lambs (Figure 4). FEC and IgA level are presented in more detail in Figure 5 while the details for each trait at each time point are given in Table S2.

As expected, no *T. circumcincta* eggs were detected in the faeces of the control animals at any time point while in the experimental animals no eggs were scored before 7 dpi (Figure 4A and Figure 5A). After 21 dpi the mean FEC was at its highest level and it

appeared to decrease with time (r= -0.81, p= 0.05, df= 4 time points). Worm egg production was negatively correlated with body weight. A 5 kg difference in weight (25 to 30 kg) was associated with a 80 eggs per gram increase in FEC (50 to 130 eggs per gram, df= 47, $p<0.001$) after having corrected for the host age. An infection of coccidian parasites was also detected and this was most prominent when the lambs were younger than 4 months (35 dpi in Figure 4B); it was then controlled by veterinary treatment (Table S1). No convincing difference in coccidia oocysts count was found between control and experimental animals.

In order to determine the reliability of FEC as a measure of infection, we investigated its correlation with the actual parasite burden found after *post mortem* examination. As mentioned above, FEC is a good predictor of the actual burden (Figure 2C). However, this association between FEC and adult worm count became progressively weaker when earlier FEC points were tested (rs=0.83, 0.79, 0.56, 0.46, 0.29, 0.17 at 91, 77, 63, 49, 35, 21 dpi, df= 45) and was not significant 50 days before *post mortem* count (49 dpi). Despite the general agreement between FEC and *post mortem* count, five animals had a worm burden between 400 and 2400 but very low (undetectable) faecal eggs.

The trait that showed the clearest correlation with the degree of infection was the IgA level (Figure 4C). The difference between control and experimental animals was significant throughout the infection including, surprisingly, the very beginning of the experimental infection (0 dpi) when no faecal eggs were detected (Figure 5B). IgA level in control lambs did not co-vary with any explanatory variable and did not show any temporal trend thus suggesting that the IgA measured is the background level. In contrast, IgA level in experimental animals increased constantly with time and decreased with increasing FEC. The correlation with FEC changed from r=-0.17 (NS, df= 45) at 14 dpi to r=-0.44 at 84 dpi ($p<0.001$, df= 45). In addition, the IgA level measured two weeks before sacrifice (84 dpi) was negatively correlated with adult worm count (Spearman's rho= -0.42, $p<0.01$, df= 45).

The effect of the infection was not always obvious on the concentration of white blood cells (Figure 4D-I). The total white blood cells (WBC) were consistently higher in the experimental animals than in the controls 3although this difference was not significant (Figure 4D). However, the co-variation with FEC was positive and significant at p=0.006 (r=0.64, df= 45). After a peak at 14 dpi, WBC decreased with time but the same pattern occurred in the control animals so the relationship before WBC with FEC is unclear. A similar picture appeared for lymphocyte, monocyte and neutrophils where the correlations with FEC were positive and significant but the difference compared to the controls was not significant (Figure 4G-I) while basophils did not vary with FEC. The eosinophil concentration (Figure 4F) was significantly different between controls and infected ($p<0.01$). Body weight did not differ significantly between controls and infected although the latter were always lighter by between 0.55 (2 dpi) and 2.2 kg (86 dpi). Scaled by the average weight of the infected lambs, this means that infected sheep were between 3 (2 dpi) and 7% (86 dpi) lighter than controls. The effect of infection (FEC) on weight was mild but significant and resulted in a reduction of weight of approximately 0.06 kg/100 faecal eggs per gram ($p<0.0001$) throughout the experimental period

3.3 Association between phenotypic variation and estimated breeding values

Association with maternal estimated breeding values were investigated by comparing lambs in the two dam breeding value groups (Materials and Methods). Lambs born from the resistant group of dams had fewer adult worms than those born from the susceptible group (1755 vs 2669, Figure 6A); although substantial, this difference was not significant (p=0.21, df=45, Mann-Whitney test). However, the breeding values were estimated for lamb FEC and, consistently, lambs born from the resistant dams had lower FEC at each sampling point. At the last FEC, this difference was 98 vs 264 eggs per gram ($p<0.05$, df=45, Mann-Whitney

test, Figure 6B). The difference between groups in the IgA level appeared to diverge with time and was significant from 56 dpi onwards (Figure 6C and Figure 7). Interestingly, a remarkable difference in body weight was detected between groups, the resistant group was 1.9 ± 0.8 kg (p=0.02, df= 45, t-test) heavier than the susceptible group (Figure 6D) and this difference was also observed within the control group (34.2 vs. 31.7 kg at 86 dpi) but was not significant (p=0.24, df=8, t-test).

4. Discussion

The aim of this project was to study the relationship between host and parasite in naïve lambs experimentally trickle infected with *T. circumcincta*. The experiment was designed to simulate a typical infection of *T. circumcincta* in outdoor grazing conditions. The advantage of this study is that it allowed a systematic comparison between infected and control sheep grown under similar conditions. The intensity of infection was in the same range as that reported in literature. For example, in naturally infected Blackface lambs in Scotland it is usual to find between 1500 and 6500 adult worms per lamb and FEC ranging from zero to approximately 1000 eggs per gram with mean around 300 (Stear et al., 2006). Similar values are reported in Davies et al. (2006) again in naturally infected Blackface lambs. Given the wide distribution that characterizes worm and faecal egg count in typical surveys, it seems reasonable to assume that the experiment reported here was a reliable simulation of outdoor, natural infection of *T. circumcincta*.

From our results, we suggest that a host's resistance is associated with its ability to produce high levels of IgA and eosinophils although at this stage it is not possible to infer any causal correlation between these variables. Host resistance seems to cause a reduction in the total number of adults in the worm population but not a significant change in the number of EAL4 worms which, in terms of frequency, become the most represented in resistant sheep. To aid

the biological interpretation of the IgA assays, it is useful to note that the likely site of action of IgA is the abomasal mucosa, and Henderson and Stear (2006) found a correlation between plasma and mucosal IgA concentration of 0.66 ($p<0.01$) in Blackface sheep infected with *T. circumcincta*. Lambs with fewer adult worms had lower FEC (Figure 2C and Figure 8A) and higher levels of IgA (Figure 8B). However, while adult worms were the most abundant and highly aggregated in relatively few lambs, EAL4 were less abundant but present in virtually all of the lambs (Table 1). EAL4 were particularly over-represented in lambs with zero FEC, (Figure 3A and B). Other studies (Stear et al., 1999; Strain et al., 2002; Henderson and Stear, 2006) report a correlation between IgA level and worm length although they do not mention a correlation with adult worm burden.

It has been proposed that resistance to mixed strongyle infection dominated by *T. circumcincta* is acquired and not innate (Stear et al. 1999) on the basis that the heritability of FEC in lambs younger than two months is essentially null, and increases with age up to 0.33 in 6 month old lambs (Stear et al., 1996). Our results, based on phenotypic and genetic evidence, seem to support this hypothesis. The difference in IgA levels and FEC between high and low infected lambs was evident only after 56 dpi (Figure 8A-B). Lambs become less susceptible, presumably as a result of developments in their acquired immune response as they are exposed to infection. Indeed, this was reflected by IgA levels rising continuously with time while the prevalence of FEC decreased with time from 89% (21 dpi) to 57% (91 dpi, Table 3). This implies also that naïve lambs will be colonized by *T. circumcincta* as soon as the lambs are exposed to normally infected pastures, and before the immune system is able to counteract the infection, *T. circumcincta* will have completed at least one life cycle. In this respect, it also interesting to note that the highest FEC was reached 21 days after the first challenge with infective larvae, and this is the time required by the larvae to mature, copulate and lay eggs (Figure 4A and 5A).

Some insights can be gained from this study about the effect of selection for resistance. As explained in Materials and Methods, the lambs here studied derived from parents with diverse genetic resistance to *T. circumcincta*. Although the target trait for assessing genetic resistance was FEC, it appears that the estimated breeding values were associated more with body weight and IgA rather than on the *post mortem* worm burden or FEC itself. Therefore, IgA level may be a target trait to complement FEC in breeding programs. The IgA level could be more sensitive than FEC in detecting infection since sheep having no detectable eggs could be identified from their IgA level. In addition, the heritability of IgA activity was found to be higher (0.46-0.67) than that of FEC (0.33) thus offering a faster response to selection (Davies et al., 2005). It should be noted that it is not possible to determine to what extent the phenotypic variation observed in this work was due to additive genetics effects or maternal effects. Maternal effects can be substantial at the very early stages of life (Wilson et al., 2005) and failing to account for them may reduce the effect of selective breeding.

The IgA level in experimental lambs was higher than that of the control lambs at the beginning of the experimental infection. No differences in genetic background, accidental infections or treatments between control and infected lambs (see Materials and Methods) could easily explain this bias. This might imply that a natural infection of *T. circumcincta* occurred in the experimental group which was separated from the control group before first infection, for practical reasons. However, no eggs could be detected before 21 days post (experimental) infection. The validity of the results should not be affected since the IgA level in control lambs was always low and constant with time. This is an additional line of evidence to suggest that the IgA level is a highly sensitive indicator of infection.

It is known, and expected, that a consequence of parasitism is a loss of body weight, although few quantitative studies have been published in this respect (Coop et al., 1985).

In our study, control lambs were always heavier and tended to gain weight faster but not significantly so. Eight-six days post infection, infected lambs were 2.2 kg or 7% lighter than control lambs. It seems reasonable to assume that the lack of significance was due to the small sample size rather than to a negligible effect. Interestingly, among infected lambs, the breeding value group with higher genetic merit was 1.9 kg heavier than the susceptible group, a non-negligible difference if compared with the above difference between infected and control lambs. Even within control lambs, the difference between groups was 2.5 kg in favour of the resistant group, albeit not significant. Within infected lambs, the relationship between FEC and weight was negative thus confirming that parasite infection slows down the growth of the host. Further work would be desirable to explore the genetic basis of selection for lower FEC and its relationship with IgA and body weight.

No evidence was found for density dependent fecundity of *T. circumcincta* in the present study. The severity of infection reached in this study might not have been high enough to depress worm fecundity. Bishop and Stear (Bishop and Stear, 2000) found the highest FEC between, approximately, 1000 and 5000 adult worms per sheep with a peak at 2200 worms/lamb (FEC about 280). In the present study, the mean intensity of adult worms was about 2800 (Table 1) and therefore we would expect that the egg output was at its highest level (Figure 5A). The sex ratios in adult worms were biased towards females (55:45) and a similar observation was made in Soay sheep (Craig et al., 2006). It would be interesting to establish how factors such as parasite density or host immune-response influence this ratio (Dineen and Windon, 1980; Gruner et al., 1994; Balic et al., 2000). Unfortunately, the sample size of the present study did not allow such an investigation.

This experiment was part of a larger project aimed at identifying the genetic basis of parasite resistance. The phenotypic data presented here will be combined with gene expression

analysis and, potentially, genotypic characteristics in order to provide a comprehensive picture of the mechanisms leading from genotype to phenotype.

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Table 1. Description of worm abundance

Worm stage	% of worm pop.[a]	Prevalence (95% ci)[b]	Intensity (95% ci)[c]
EAL4	10.3	0.98 (0.94-1.00)	318 (254-402)
L4	11.2	0.87 (0.77-0.96)	386 (299-498)
L5	6.0	0.70 (0.57-0.83)	258 (185-344)
Adult female	39.8	0.79 (0.68-0.89)	1523 (1146-2016)
Adult male	32.7	0.79 (0.68-0.89)	1253 (922-1663)
Adult total	72.5	0.79 (0.66-0.89)	2773 (2108-3736)

[a] Percentage as: count of life stage/ total worm population (all the worms across all the lambs). [b] Percentage of infected lambs. [c] Mean number of parasites in infected lambs only (lambs with count of zero excluded). Note that the mean abundance (mean number of parasite over all lambs with and without count of zero) is Prevalence x Intensity.

Table 2. Worm size and fecundity.

Worm population average[a]				
	n	**Mean (95% ci)**	**Range**	**CV**[b]
Length (mm)	337 worms	8.42 (8.30-8.53)	4.65-10.93	12.46
Egg count	632	20.36 (19.45-21.27)	0-59	57.03
Lamb average				
Length (mm)	35 lambs	8.47 (8.23-8.71)	6.63-9.85	8.24
Egg count	37	19.87 (17.41-22.33)	4.90-34.70	37.09

[a] The worm population considered as all the worms across all the lambs. b CV, coefficient of variation (standard deviation/$n^{1/2}$ x 100).

Table 3. FEC abundance at different time points.

dpi[a] (age weeks)	Prevalence (95% ci)[b]	Intensity (95% ci)[c]
21 (16)	0.89 (0.79-0.98)	554 (440-688)
35 (18)	0.89 (0.81-0.98)	360 (277-479)
49 (20)	0.87 (0.77-0.96)	542 (386-940)
63 (22)	0.68 (0.53-0.81)	165 (115-223)
77 (24)	0.66 (0.53-0.79)	288 (198-466)
91 (26)	0.57 (0.45-0.72)	220 (160-329)

[a] dpi: days post infection. [b] Percentage of infected lambs. [c] Mean number of parasites in 560 infected lambs only (lambs with count of zero excluded). Note that the mean abundance 561 (mean number of parasite over all lambs with and without count of zero) is Prevalence x 562 Intensity.

Legends to Figures

Figure 1. Calendar of sampling for the data shown on the left hand side (PM: ascertainment of post mortem worm burden) with dpi shown on top of each point. The dashed line marks the beginning of the experimental infection, i.e. 0 dpi (the time-course graphs shown elsewhere start from this point).

Figure 2. Worm burden quantified after post mortem examination. A) Box-plot of worm stages. Boxes span the interquartile range, comprising approximately half of the data- points, with median values marked by horizontal lines; vertical lines extend to include 95% of the distribution. Circles are outliers. B) Lambs ranked by adult worm burden (thin lines) with FEC measured at the last sampling point overimposed (thick lines). C) Adult worm burden grouped in classes of abundance (x-axis labels: mid-class value) plotted against the corresponding FEC at final sampling. Bars are standard errors. D) Worm fecundity as function of worm length (egg=-45.6+ 7.8(length); R2= 0.57, 579 $p<0.001$).

Figure 3. Comparison between worm burden in sheep that scored FEC>0 and FEC=0 at the last sampling before sacrifice. A) Average of each life stage. B) Average frequency of each life stage. Error bars are standard errors of the means.

Figure 4. Mean value of each trait plotted against time (days post infection). Solid lines are the means of infected lambs, dashed lines are control lambs. Error bars have been omitted for ease of readability (Table S2). Means significantly different between control and infected groups are marked by asterisks (* $p<0.05$, **$p<0.01$, ***$p<0.001$). Trait names are reported on y-axes.

Figure 5. Box-plots for selected longitudinal traits over time. A) FEC. B) IgA level. See legend of Figure 2A for interpretation of box-plots.

Figure 6. Comparison between breeding value groups at the last sampling (Res., resistant group, n=25; Sus., susceptible group, n=22). The mean of each group and the significance of the differences between resistant vs. susceptible are: A) 1755 vs 2669 worms/lambs, NS. B) 98 vs. 294 eggs/lambs, $p<0.05$. C) 0.56 vs 0.32 IgA relative concentration, $p<0.05$. D) 32.5 vs 28.8 kg/lamb, $p<0.01$. See legend of Figure 2A for interpretation of box-plots.

Figure 7. IgA concentration in the resistant (solid line) and susceptible (dashed line) breeding value groups over time. Asterisks mark significant differences between resistant and susceptible ($p<0.05$ after Wilcox test at each time point).

Figure 8. Comparison of FEC and IgA between the ten most infected and ten least 606 infected lambs. The criterion chosen to quantify infection was the burden of adult worms 607 counted post mortem. This figure illustrates the main features of resistance and 608 susceptibility. Vertical bars are standard errors.

Fig.1

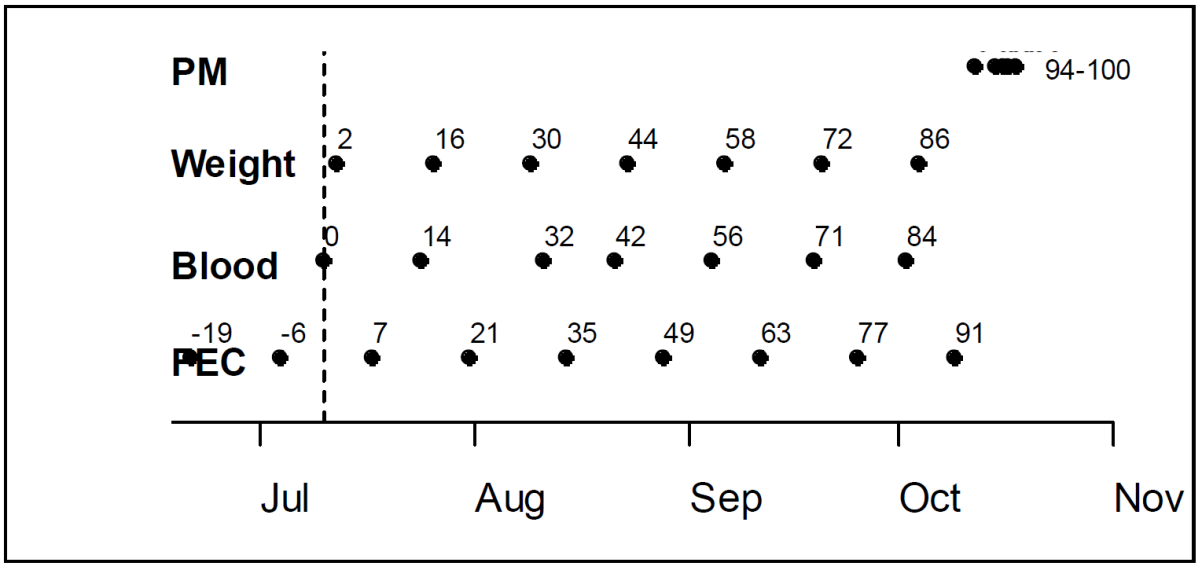

PM
94-100
Weight
2
16
30
44
58
72
86
Blood
0
14
32
42
56
71
84
PEC
-19
-6
7
21
35
49
63
77
91
Jul
Aug
Sep
Oct
Nov

Fig.2

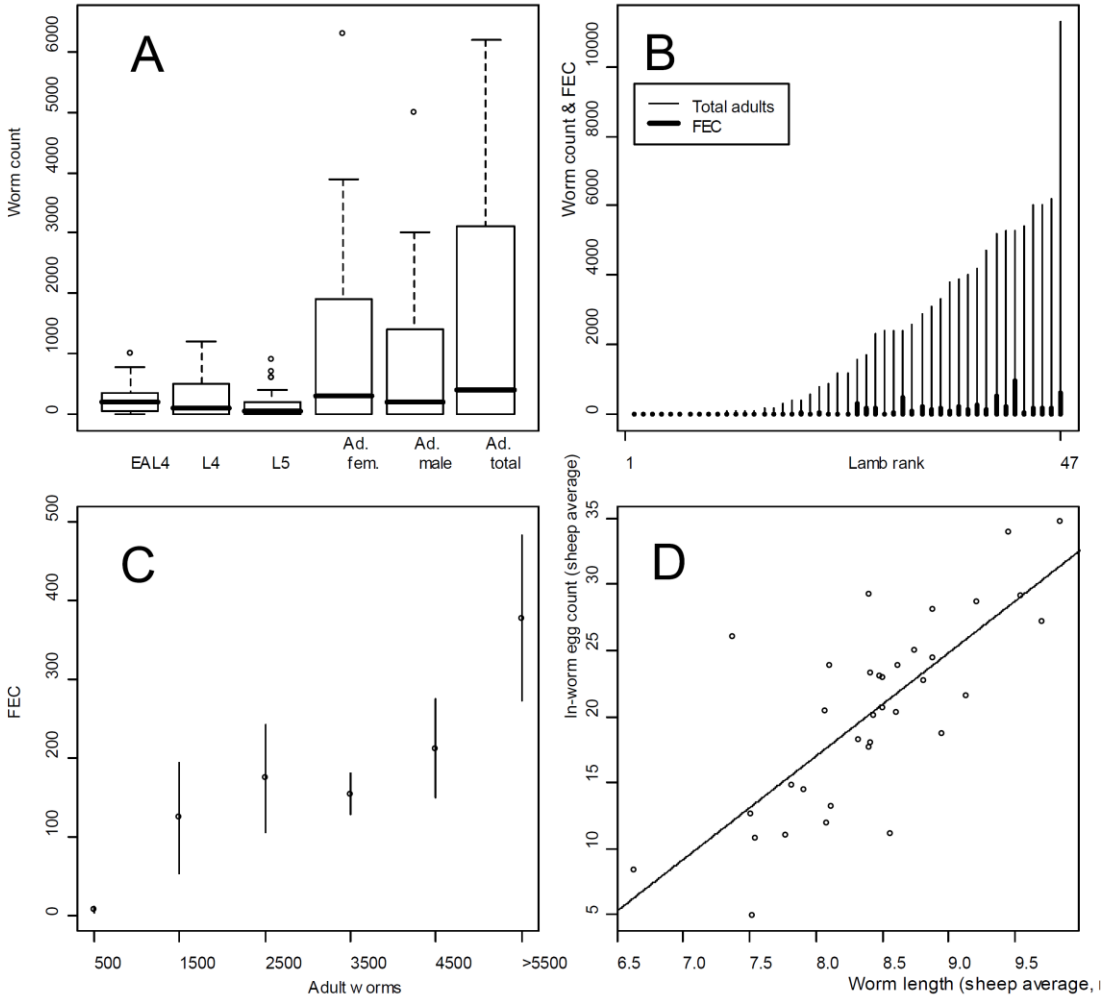

A
Worm count
0
1000
2000
3000
4000
5000
6000
EAL4
L4
L5
Ad. fem.
Ad. male
Ad. total
B
Worm count & FEC
0
2000
4000
6000
8000
10000
Total adults
FEC
1
Lamb rank
47
C
FEC
0
100
200
300
400
500
500
1500
2500
3500
4500
>5500
Adult worms
D
ln-worm egg count (sheep average)
5
10
15
20
25
30
35
6.5
7.0
7.5
8.0
8.5
9.0
9.5
Worm length (sheep average,

Fig.3

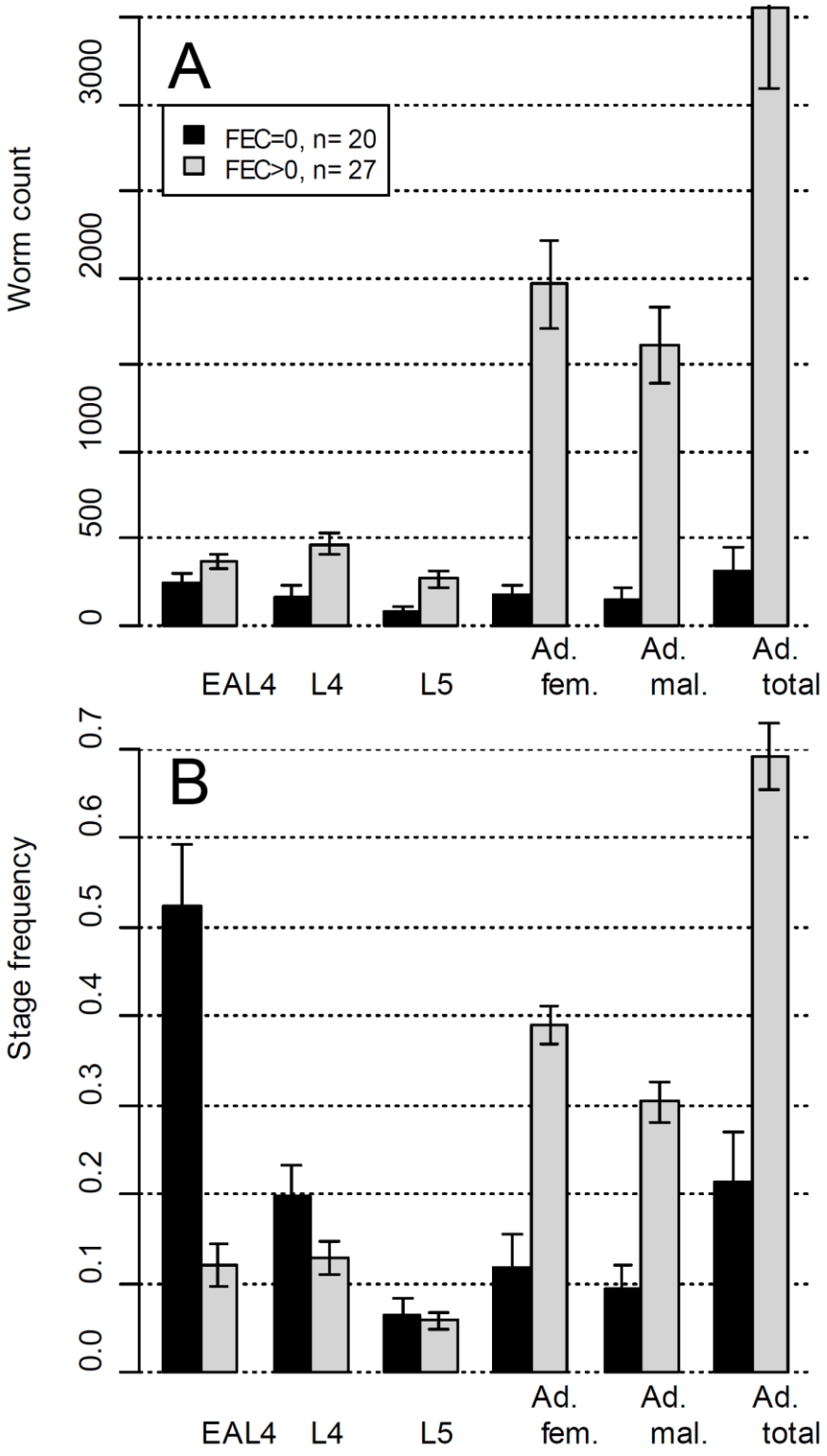
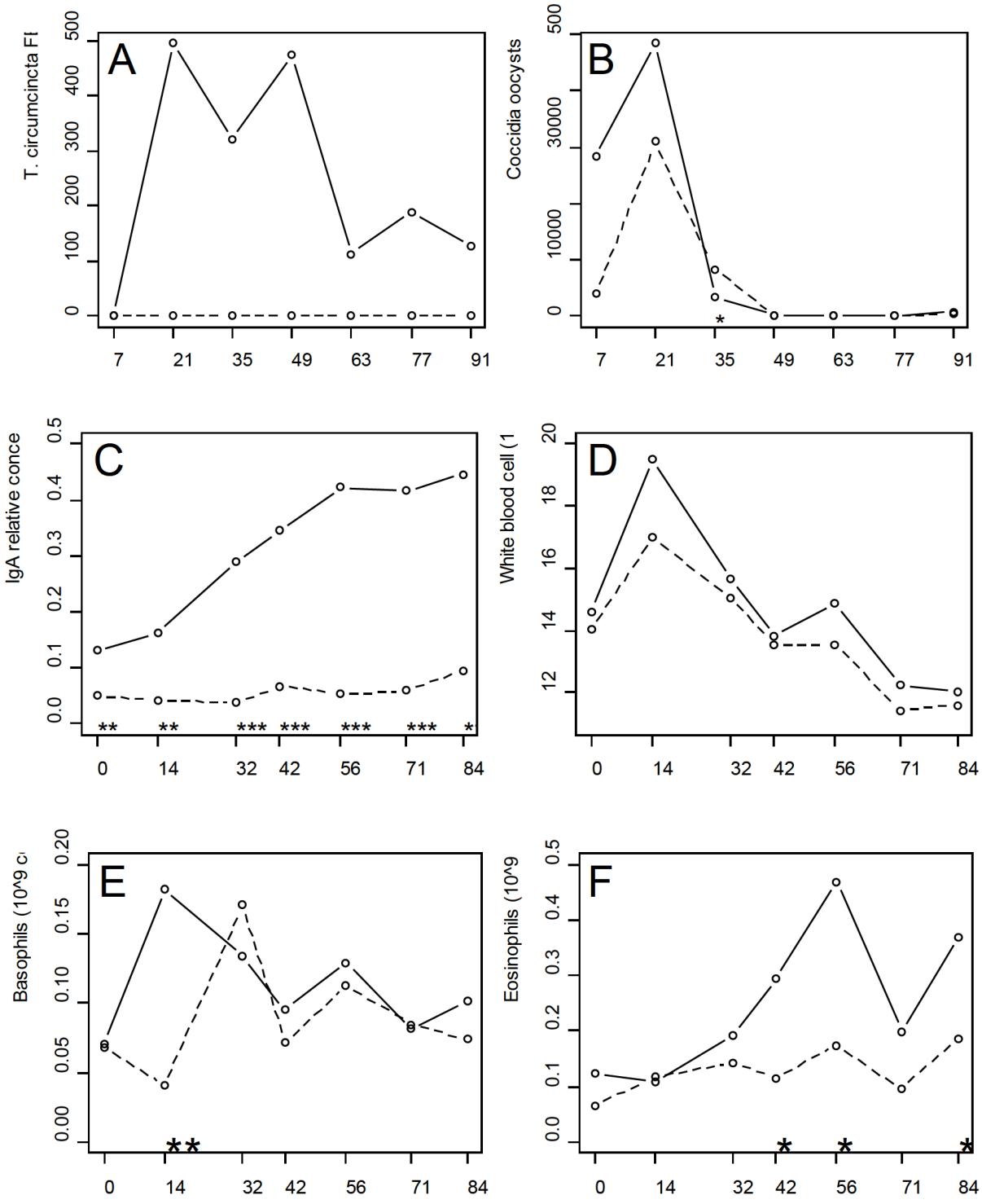

A
FEC=0, n= 20
FEC>0, n= 27
Worm count
0
500
1000
2000
3000
EAL4
L4
L5
Ad. fem.
Ad. mal.
Ad. total
B
Stage frequency
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
EAL4
L4
L5
Ad. fem.
Ad. mal.
Ad. total

Fig.4


A
T. circumcincta FI
0
100
200
300
400
500
7
21
35
49
63
77
91
B
Coccidia oocysts
0
10000
30000
500
*
C
IgA relative conce
0.0
0.1
0.2
0.3
0.4
0.5
**
**
***
***
***
***
*
0
14
32
42
56
71
84
D
White blood cell (1
12
14
16
18
20
E
Basophils (10^9 c
0.00
0.05
0.10
0.15
0.20
**
F
Eosinophils (10^9
0.0
0.1
0.2
0.3
0.4
0.5
*
*
*

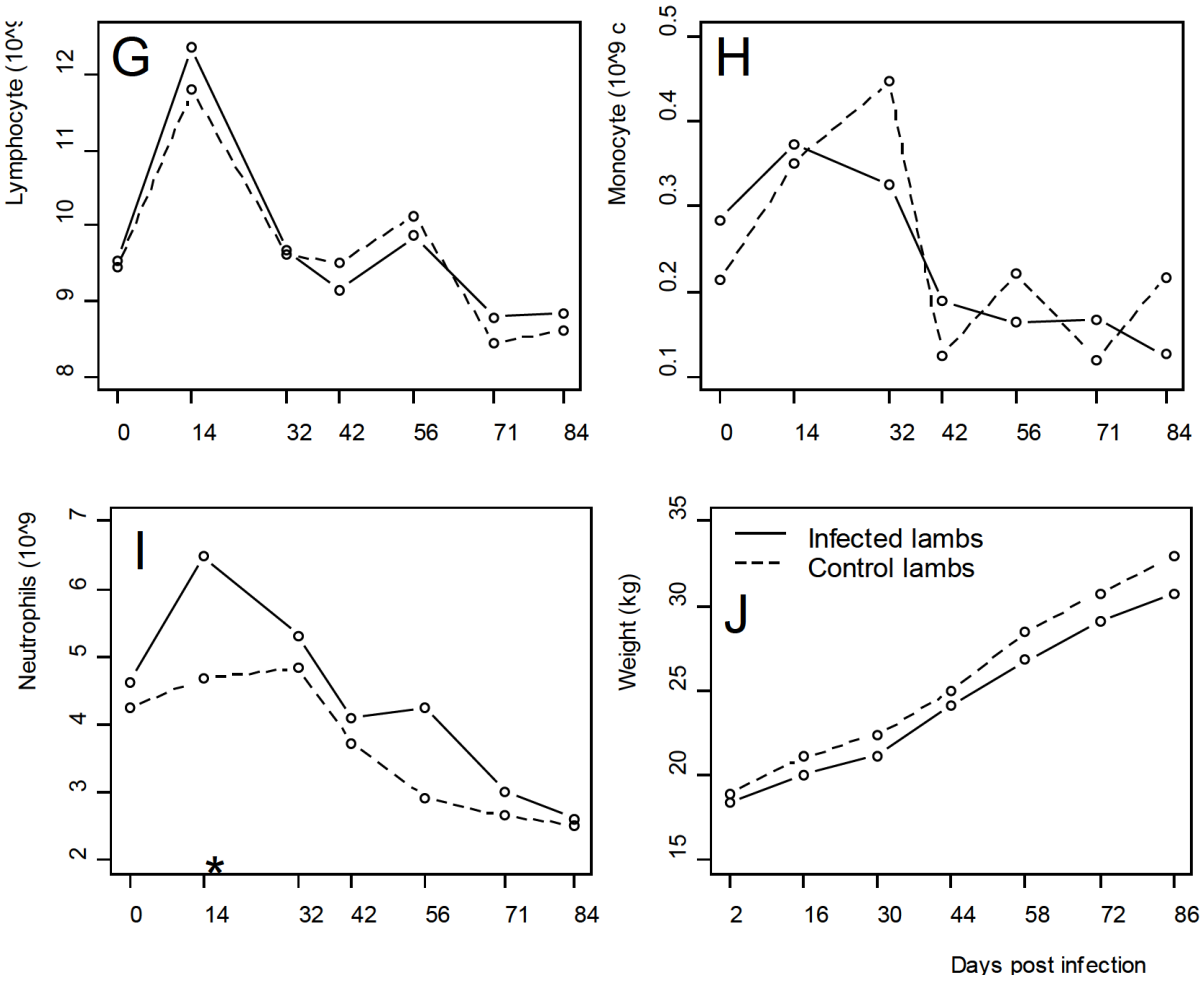
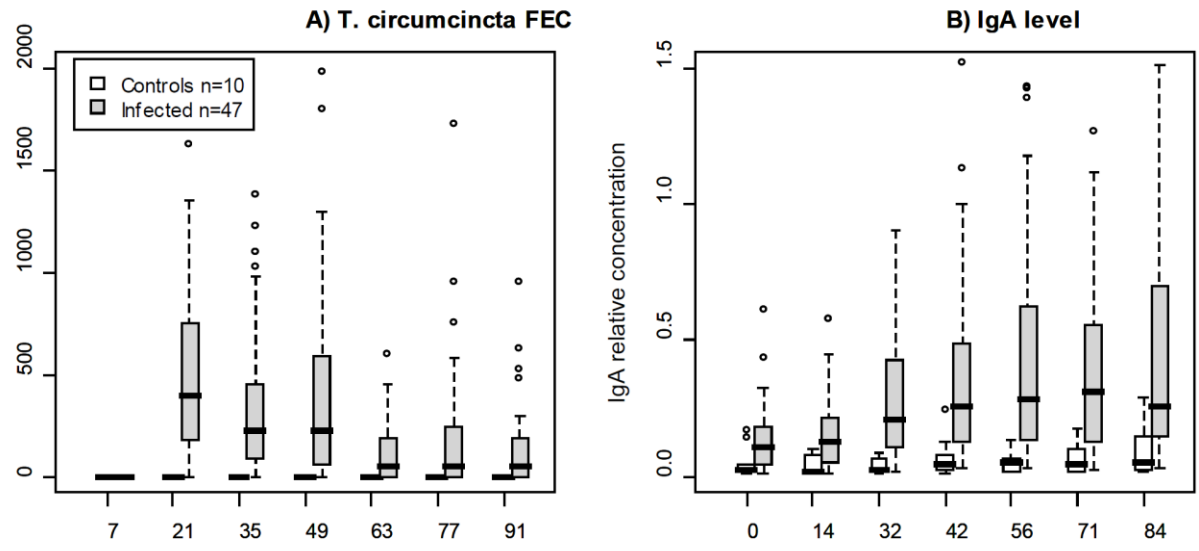

G
Lymphocyte (10^9
8
9
10
11
12
0
14
32
42
56
71
84
H
Monocyte (10^9 c
0.1
0.2
0.3
0.4
0.5
I
Neutrophils (10^9
2
3
4
5
6
7
*
J
Weight (kg)
15
20
25
30
35
Infected lambs
Control lambs
2
16
30
44
58
72
86
Days post infection

Fig.5



Days post infection

Fig.6

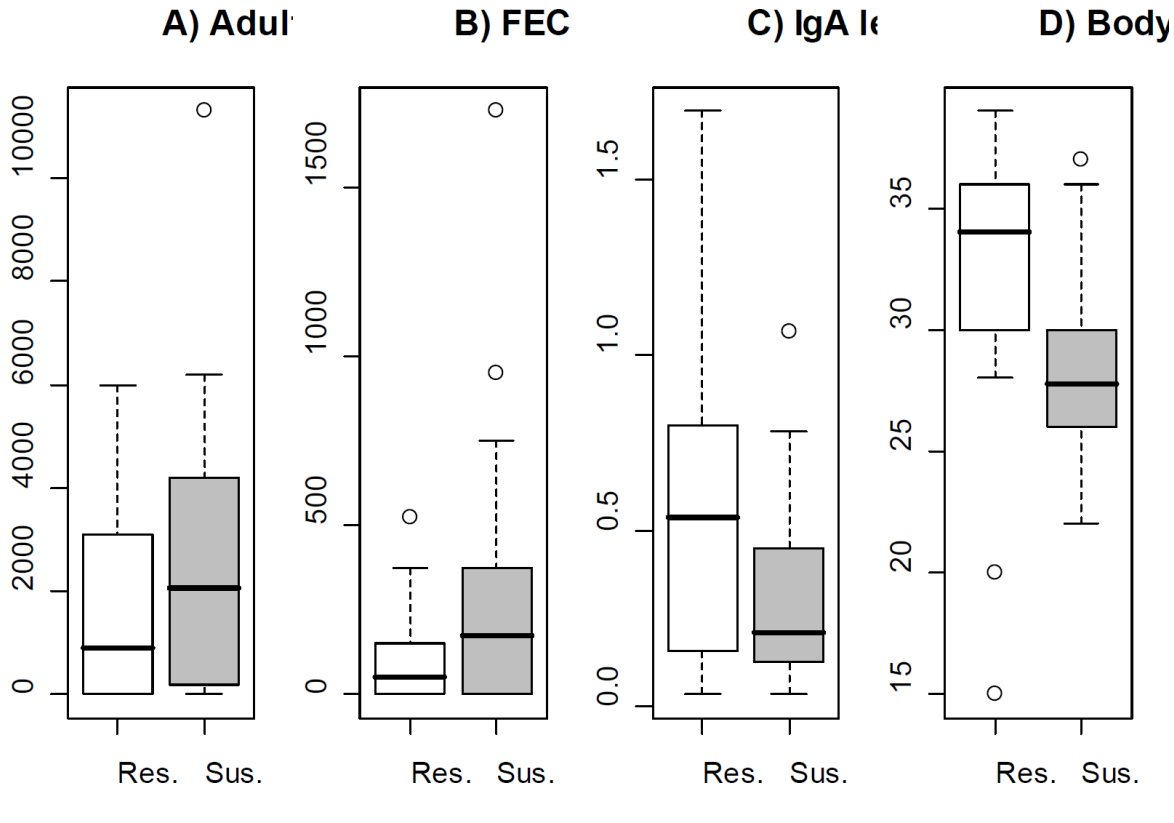


Fig.7

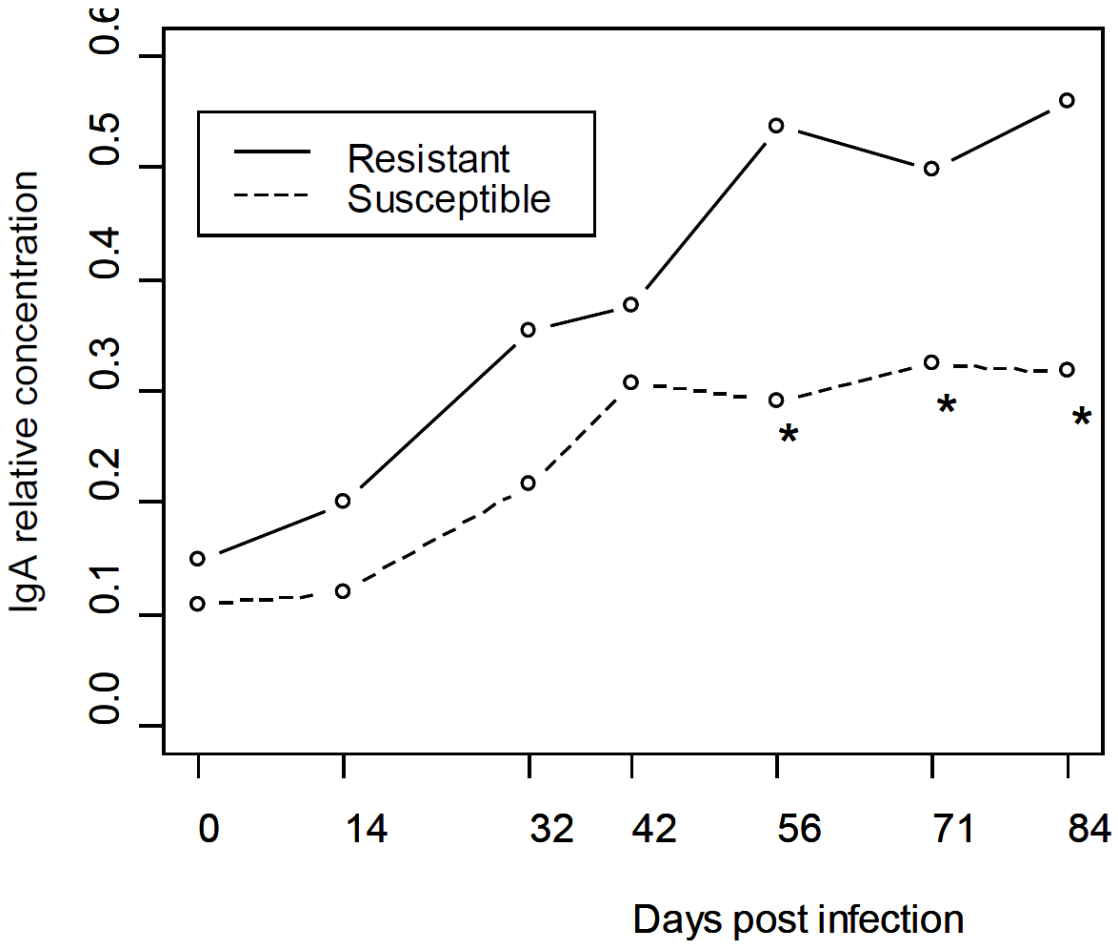
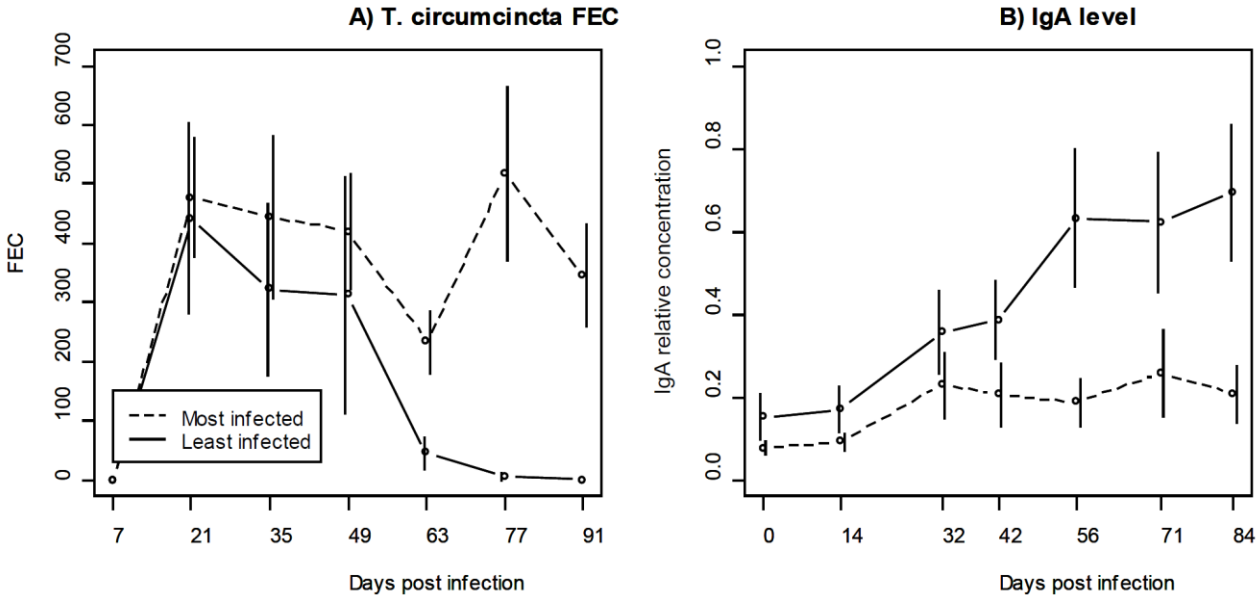

IgA relative concentration
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Resistant
Susceptible
*
*
*
0
14
32
42
56
71
84
Days post infection

Fig.8


A) T. circumcincta FEC
FEC
0
100
200
300
400
500
600
700
Most infected
Least infected
7
21
35
49
63
77
91
Days post infection
B) IgA level
IgA relative concentration
0.0
0.2
0.4
0.6
0.8
1.0
0
14
32
42
56
71
84
Days post infection