The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep

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SUMMARY

Previous studies in deliberately infected sheep have shown an association between IgA activity against 4th-stage larvae of *Teladorsagia circumcincta* and parasite growth, development and fecundity. The purpose of this research was to determine if these results could be confirmed in naturally infected sheep and to explore the hypothesis that plasma IgA activity could help to identify resistant lambs with shorter adult nematodes. Plasma IgA activity was skewed with most animals having relatively low levels of IgA activity. Plasma IgA activity was repeatable and highly heritable. Animals with increased IgA activity had lower egg counts and shorter adult female *T. circumcincta*. Therefore, under conditions of natural parasite challenge, plasma IgA activity may help to identify lambs resistant to *T. circumcincta*.

Key words: sheep, nematoda, *Teladorsagia circumcincta*, IgA, marker, indicator trait.

INTRODUCTION

The resistance of sheep to infection with the parasitic nematode *Teladorsagia circumcincta* depends upon the age and the history of exposure of the host. In the UK, the development of resistance in spring-born sheep grazing naturally infected pasture goes through at least 5 stages: pre-weaning (Stear *et al.* 1995*a*), post-weaning (Waller & Thomas, 1978; Stear, Park & Bishop, 1996; Moskwa *et al.* 1998), winter (Stear *et al.* 2000*a*), adulthood (Stear *et al.* 2000*b*) and the periparturient period (Connan, 1968). In addition to the dynamic variation over time, sex (Barger, 1993; Stear *et al.* 1996), type of birth (Stear *et al.* 1996), extent of early exposure (Stear *et al.* 1996), genetics (Bishop *et al.* 1996), maternal effects (Bishop *et al.* 1996) and nutritional status (Coop, Huntley & Smith, 1995; Stear *et al.* 2000*a*) also affect host resistance to *T. circumcincta* infection. In the UK, most research on resistance to *T. circumcincta* has involved weaned lambs because these animals suffer most from the subclinical effects of infection. The major manifestation of resistance in these animals appears to be the inhibition or retardation of nematode growth and development (Stear *et al.* 1997).

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Smith *et al.* (1985) reported an extremely strong association between variation in IgA responses and the length of *T. circumcincta* recovered after deliberate infection. Further research has confirmed and extended this result (Stear *et al.* 1995*a*). Specifically, increased abomasal IgA activity against 4th-stage larvae was associated with both increased larval inhibition and decreased adult size. In addition, the length of adult female *T. circumcincta* is strongly associated with the number of eggs *in utero* and the number of eggs laid (i.e. worm fecundity) (Stear *et al.* 1995*a*; Stear & Bishop, 1999).

The strength of the association between IgA and parasite fecundity led us to hypothesize that the specificity and activity of local IgA was the major mechanism regulating the fecundity of *T. circumcincta* (Stear *et al.* 1996) and a major mechanism of resistance to infection in lambs (Stear *et al.* 1995*a*; McCririe *et al.* 1997; Stear *et al.* 1997; Strain & Stear, 1999). Additional mechanisms of resistance may also operate, especially in older animals, such as immediate hypersensitivity reactions against incoming larvae or established worms (Miller & Huntley, 1982; Miller *et al.* 1983; Stear *et al.* 1995*a*; Miller, 1996).

This study was designed to test whether naturally-infected grazing lambs showed an association between parasite-specific IgA and adult worm length and whether IgA could be used as an indicator trait to identify sheep with greater degrees of resistance or susceptibility.

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MATERIALS AND METHODS

Animals

Data were collected each year for 5 years (1992–1996) from consecutive cohorts of 200 lambs. All lambs were Scottish Blackface sheep from a commercial upland farm in southwest Strathclyde. The dominant nematode on this farm is *T. circumcincta* (Stear *et al*. 1998). Husbandry procedures followed normal, commercial practice and have been described previously (Stear *et al*. 1998). Briefly, all lambs were born outside and were continuously exposed to mixed nematode infections by grazing. Every 28 days from 4 to 24 weeks of age, all lambs were treated with a broad-spectrum anthelmintic, given according to the manufacturer's recommendation. Six or 7 weeks after the final anthelmintic treatment, about one-half of the lambs in 1992–1995 were slaughtered at the local abattoir. At slaughter the lambs were 6–7 months of age. Blood samples were collected in August, September and October every year with the exception of October 1992, October 1993 and August 1995.

Parasitology

Faecal egg counts were estimated by the McMaster technique (Gordon & Whitlock, 1939). Each egg counted represented 50 eggs per gram of faeces. At slaughter the abomasum was removed, opened along the greater curvature and washed with tap water under moderate pressure. The contents and washings were made up to 2 l, from which ten 4 ml aliquots were examined to estimate the size of the adult nematode population. The mucosa from one half of the washed abomasum was digested with pepsin-HCl for 6 h at 42 °C; the digest was then made up to 2 l to estimate the number of larvae (Armour, Jarrett & Jennings, 1966).

Standard procedures were used to estimate the mean length of adult female nematodes (Stear *et al*. 1997). The mean length (mm) was estimated by image analysis of at least 25 female adult worms from each sheep.

IgA activity

The activity of plasma IgA against a somatic extract of 4th-stage larvae from *T. circumcincta* was measured by indirect ELISA. Fourth-stage larvae were harvested 4 days after infecting helminth-naïve lambs with 150000 infective larvae. The abomasum was washed with tap water and cut into strips. These strips were suspended in Baermann funnels containing PBS (pH 7·4) at 37 °C. The larvae were then placed onto surgical swabs and the migrating larvae were recovered in PBS. These larvae were washed 5× in PBS, once in PBS containing 100 i.u. penicillin/ml, 0·1 mg streptomycin/ml, 2·5 μg amphotericin B/ml, and 0·05 mg gentamicin/ml and once in Tris-inhibitor solution (pH 8·3; 10 mM Tris containing 1 mM EDTA (disodium ethylene diamine tetraacetic acid), 1 mM EGTA (ethylene glycol *bis* (2-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 1 mM NEM (*N*-ethylmaleimide), 0·1 μM pepstatin, 1 mM PMSF (phenyl methyl sulphonyl fluoride) and 0·1 mM TPCK (*N*-tosylamide-L- phenylalanine chloromethyl ketone). After centrifugation, the pellet was resuspended in 1% sodium deoxycholate (v/v) in Tris-inhibitor solution and stored at −20 °C. After thawing, the sample was homogenized on ice with a hand-held electric homogenizer (Janke & Kunkel IKA Labortechnik). The supernatant fraction was filtered through a 0·2 μm filter and aliquots stored at −80 °C. The protein concentration was estimated with bicinchoninic acid (Pierce) and adjusted to 5 μg/ml in 0·06 M bicarbonate buffer (pH 9·6) before use.

The wells on a flat-bottomed microtitre plate (Nunc) were coated with 100 μl of parasite solution and left overnight at 4 °C. The plate was washed 5 times in PBS–Tween (0·1% v/v Tween 20 in PBS), incubated for 2 h with 200 μl of blocking buffer (4% skimmed milk powder in PBS–Tween), then again washed 5× in PBS–Tween. Then 100 μl of plasma sample diluted 1:10 in blocking buffer were added to each of 3 wells and incubated at 37 °C for 30 min. After another 5 washes in PBS–Tween, 100 μl of a rat monoclonal anti-sheep IgA at a dilution of 1:50 in blocking buffer were added and incubated for 30 min at 37 °C. After a further 5 washes in PBS–Tween, 100 μl of goat anti-rat IgG conjugated to alkaline phosphatase at 1:1000 in blocking buffer were added and incubated for 30 min at 37 °C. After 5 final washes in PBS–Tween, 100 μl of 5-bromo-4-chloro-3-indolylphosphate (Kirkegaard & Perry Laboratories) were added and incubated for a further 30 min at 37 °C. The reaction was then read on a microplate reader at 635 nm. To estimate the IgA activity, the mean of 3 replicates from a pooled sample of helminth-naïve lambs was subtracted from the sample mean and this adjusted mean was divided by the mean of 3 replicates from a pool of high-responder lambs after subtracting the mean of the helminth naïve lambs (Sinski *et al*. 1995). Three replicates from the naïve and high responder pools were included on each plate. The pool of high responder lambs was created by combining equal quantities of plasma from 6 lambs that gave strong IgA responses following natural infection. The value for each lamb was therefore expressed as a proportion of a positive control.

Statistical analysis

IgA activity was measured in a total of 964 lambs sampled in September. These values were increased

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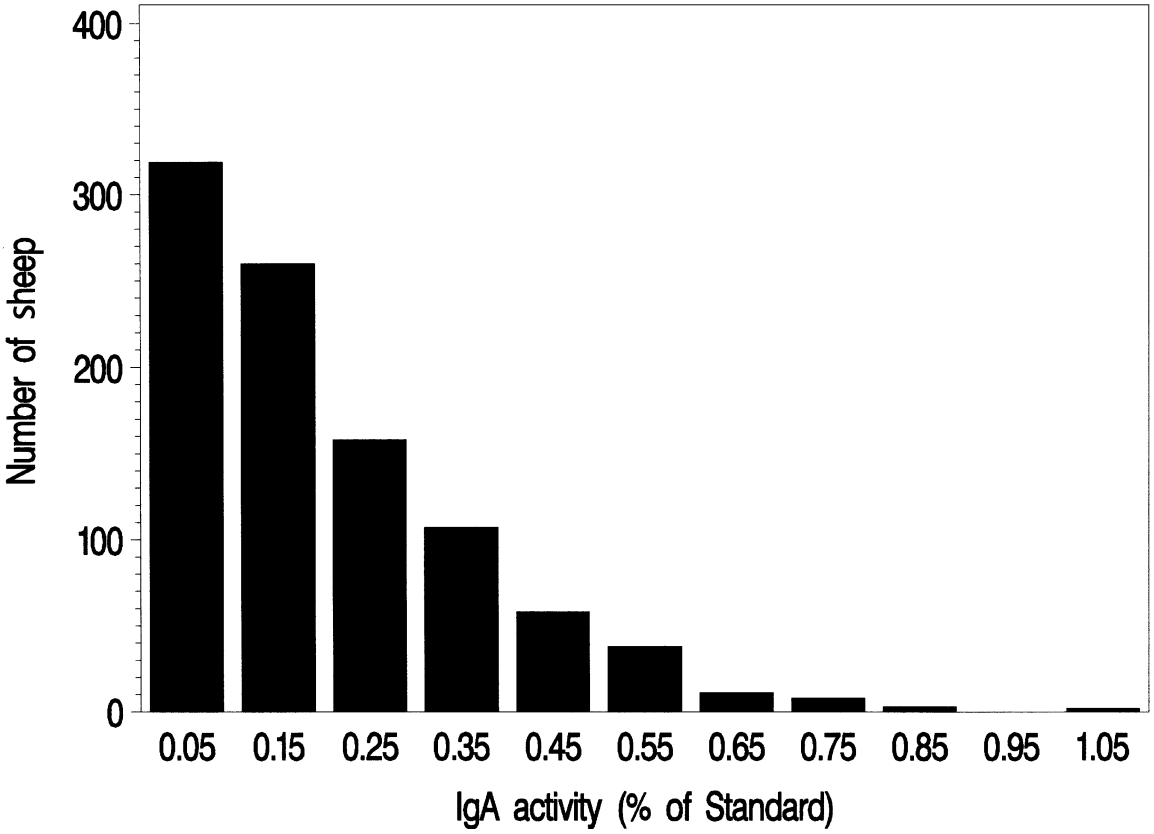

Fig. 1. The distribution of IgA activity against somatic extracts of 4th-stage larvae of *Teladorsagia circumcincta* in naturally infected lambs sampled in September.

by a value of 1 (to avoid problems with zero values), and then subjected to the Box-Cox analysis to determine an appropriate transformation (Minitab Inc., State College, Pennsylvania). The UNIVARIATE procedure in SAS (SAS Institute, Cary, N. Carolina) was used to estimate the means and variances of the transformed data. Transformed IgA activity data, using the best transformation indicated by the Box-Cox analysis (i.e. $(x+1)^{-3}$), were used in all subsequent analyses. Additionally, faecal egg counts and the numbers of adult and 4th-stage larvae of *T. circumcincta* were log-transformed (log10 $(x+1)$) prior to analyses.

A repeated measures, mixed model analysis with an autoregressive covariance structure of order 1 was applied to study the components of the variation among animals. This analysis used the MIXED procedure (SAS Institute, Cary, N. Carolina). This analysis used 2223 observations over 5 years. The fixed effects in the analysis were year, month of measurement and sex. The CORR procedure (SAS Institute, Cary, N. Carolina) was used to estimate correlations among samples collected in different months. The transformed data were used for correlation analysis.

The heritability of September IgA activity was determined with the ASREML package (www.res.bbsrc.ac.uk/pub/aar) (Gilmour *et al*. 1996) fitting an animal model and including all known pedigree relationships among animals. The fixed effects were year and sex. The lambs were the offspring of 37 sires and 491 dams.

Multiple regression analyses, using the GLM procedure (SAS Institute, Cary, N. Carolina) were used to quantify the relationship between IgA activity and parasitological variables. The mean of all covariates was adjusted to zero before analysis. For clarity, only the September results have been used to estimate the heritability of IgA activity and examine the associations with resistance to parasite infection. The results from August and October contained fewer samples but were consistent with the results and conclusions presented in this paper.

RESULTS

IgA activity against an extract of 4th-stage larvae was measured in an indirect ELISA and expressed as a percentage of a standard value. The distribution of values was positively skewed; most lambs had relatively low values but some lambs had quite high values. Fig. 1 shows the distribution of IgA activity in the 964 lambs sampled in September.

The skewed nature of the distribution of IgA activity among lambs implied that data transformation was necessary prior to variance analyses. Box-Cox analysis gave a lambda value of $-3{\cdot}034$ with a standard deviation of 0·142. Therefore all data

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Table 1. Mean (standard error) IgA activity against 4th-stage larvae of *Teladorsagia circumcincta* across sex, month and year

	August		September		October	
Year	Male	Female	Male	Female	Male	Female
1992	0·144 (0·018)	0·187 (0·018)	0·187 (0·012)	0·222 (0·016)	–	–
1993	0·118 (0·017)	0·152 (0·018)	0·221 (0·015)	0·252 (0·017)	–	–
1994	0·088 (0·009)	0·077 (0·018)	0·174 (0·015)	0·187 (0·021)	0·142 (0·012)	0·150 (0·022)
1995	–	–	0·204 (0·018)	0·246 (0·025)	0·120 (0·008)	0·154 (0·013)
1996	0·090 (0·010)	0·122 (0·013)	0·151 (0·014)	0·204 (0·017)	0·132 (0·009)	0·180 (0·015)

Table 2. Covariance analysis of number of adult *Teladorsagia circumcincta*

(The initial analysis indicated that the main effect of IgA was not significant. The remaining terms accounted for 36% of the variance. The number of adult worms and the IgA activity were transformed before analysis. The IgA transformation reversed the sign and a negative association indicates that as IgA increased the number of adult parasites also increased.)

Variable	Effect	Standard error	Probability
Intercept	3·66	0·03	0·001
Year			
1992	0·40	0·05	0·001
1993	−0·27	0·05	0·001
1994	−0·36	0·05	0·001
1995	0		
IgA * 1992	−0·51	0·21	0·015
IgA * 1993	−0·50	0·19	0·010
IgA * 1994	−0·01	0·15	0·945
IgA * 1995	0·04	0·15	0·784

Table 3. Covariance analysis of faecal egg count

(The initial analysis indicated that the interaction term was not significant. The remaining terms accounted for 26% of the variance. Faecal egg count and plasma IgA activity were measured in samples taken at the same time and both variables were transformed prior to analysis. The IgA transformation reversed the sign and the positive association indicates that as IgA activity increased the faecal egg count decreased.)

Variable	Estimate	Standard error	Probability
Intercept	2·46	0·06	0·001
Year			
1992	−0·80	0·09	0·001
1993	−0·60	0·09	0·001
1994	−1·31	0·09	0·001
1995	−0·06	0·09	0·001
1996	0		0·001
IgA	0·51	0·13	0·001

Table 4. Multiple regression analysis of the mean length of female *Teladorsagia circumcincta*

(Plasma IgA activity and the number of adult *T. circumcincta* accounted for 6% of the variance in worm length. Both variables were transformed prior to analysis. The IgA transformation reversed the sign and the positive association indicates that as IgA activity increased mean worm length decreased.)

Variable	Effect	Standard error	Probability
Intercept	0·865	0·006	0·001
IgA	0·128	0·026	0·001
Number of adults	−0·035	0·012	0·003

were transformed using the function $(x+1)^{-3}$. These transformed data gave a unimodal and symmetric distribution with a mean value of $0{\cdot}63 \pm 0{\cdot}21$. One feature of this transformation is that the order of values is reversed so that positive associations appear negative and vice-versa. This transformation was also applied to the 773 samples from August and the 565 samples from October.

Sire ($P < 0{\cdot}05$) and dam ($P < 0{\cdot}01$), as well as the fixed variables year ($P < 0{\cdot}01$), sex ($P < 0{\cdot}05$) and month of sampling ($P < 0{\cdot}001$) were all statistically associated with IgA activity against 4th-stage larvae. Table 1 represents the mean values of the untransformed data and shows that mean IgA activity was highest in September and was higher in female lambs than their castrated male contemporaries.

The estimated heritability of IgA activity in September was $0{\cdot}56 \pm 0{\cdot}11$. The correlation between transformed IgA activities was 0·55 for samples collected in August and September, 0·50 for samples collected in September and October and 0·38 for samples collected in August and October.

The geometric mean number of adult *T. circumcincta* was 3900 in the 493 lambs necropsied. There was no significant main effect of IgA activity on the log-transformed number of adult *T. circumcincta*

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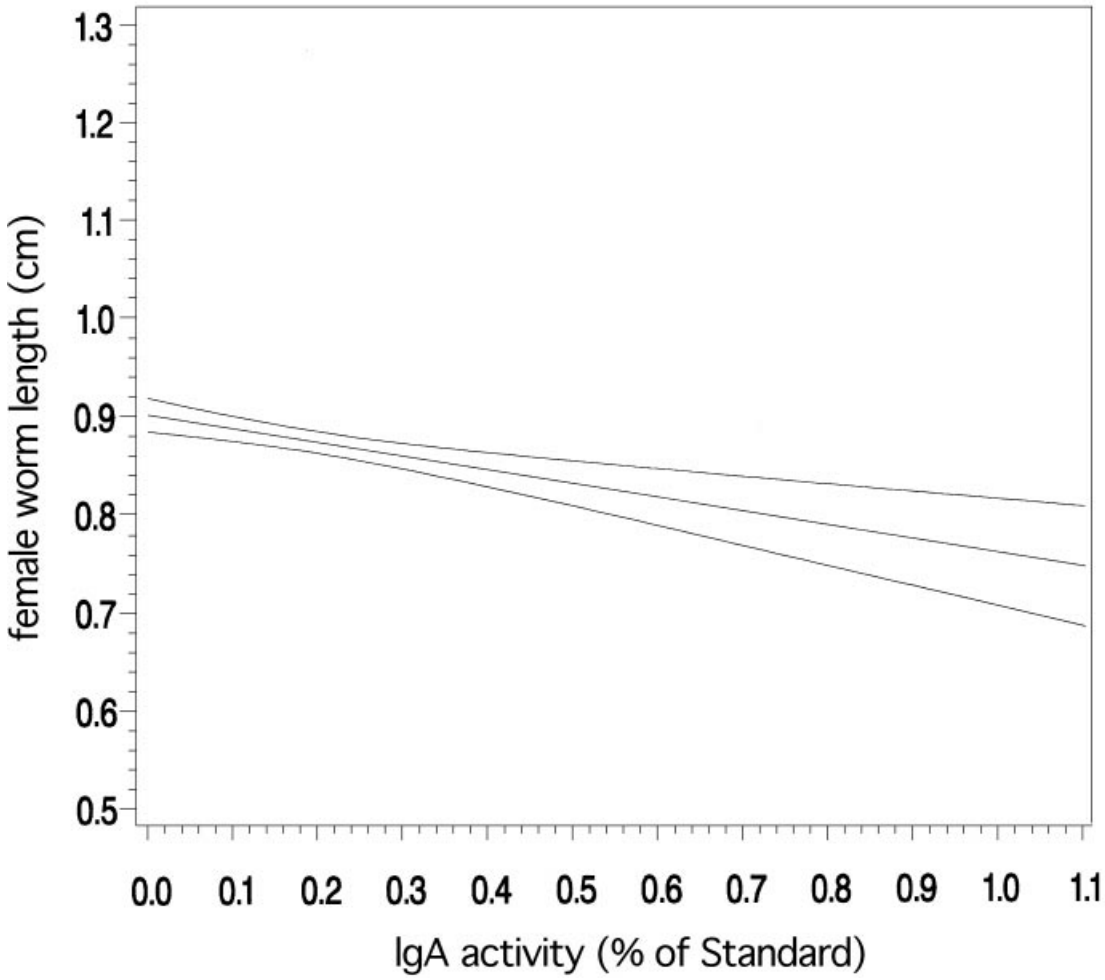

Fig. 2. The relationship between IgA activity against somatic extracts of 4th-stage larvae of *Teladorsagia circumcincta* in naturally infected lambs sampled in September and adult female worm length (cm) 6–7 weeks later.

(Table 2; $0{\cdot}70 < P < 0{\cdot}90$). However, the effect of year ($P < 0{\cdot}001$) and the interaction between year and IgA activity were significant ($0{\cdot}01 < P < 0{\cdot}05$). After back-transformation, the animals with high IgA activities in September had more parasites 6 or 7 weeks later compared to animals with low IgA activities in 1992 and 1993 but not in 1994 and 1995.

The geometric mean faecal egg count was 77 eggs per gram in September. IgA activity in September was associated with egg counts measured at the same time (Table 3). After back-transformation, increased IgA activity was associated with decreased egg counts ($P < 0{\cdot}001$). Year was also significant ($P < 0{\cdot}001$) but not the interaction between year and IgA activity ($0{\cdot}50 < P < 0{\cdot}70$). In other words, the association between IgA activity and faecal egg count was consistent across years.

The mean length of adult female *T. circumcincta* was 0·87 cm in the 491 animals examined. Two animals had too few adult worms to estimate length. Both the log-transformed number of adult *T. circumcincta* ($P < 0{\cdot}01$) and IgA activity ($P < 0{\cdot}001$) had an influence on worm length (Table 4). After back transformation, increased IgA activity and increased numbers of adult worms were associated with reduced worm length. Fig. 2 illustrates the reduction in worm length as IgA activity increased.

There appeared to be confounding between the effects of year and of number of *T. circumcincta* on worm length. When year was added to the multiple regression model, it was significant ($P < 0{\cdot}0001$) but the association with the log-transformed number of adult *T. circumcincta* was no longer significant ($0{\cdot}50 < P < 0{\cdot}70$).

The geometric mean number of 4th-stage larvae in the 493 lambs necropsied was 203. The log-transformed number of *T. circumcincta* ($P < 0{\cdot}001$) and its interaction with IgA activity ($P < 0{\cdot}001$) but not the main effect of IgA activity ($0{\cdot}50 < P < 0{\cdot}70$) were associated with the log-transformed number of 4th-stage larvae (Table 5). After back transformation, increased numbers of adult *T. circumcincta* were associated with increased numbers of 4th-stage larvae but this effect was moderated as IgA activity and adult worm numbers jointly increased.

As with worm length, there also appeared to be confounding between the effects of year and of the number of *T. circumcincta*. When year was sub-

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Table 5. Multiple regression analysis of the number of 4th-stage larvae of *Teladorsagia circumcincta*

(The initial analysis indicated that the effect of IgA was not significant. The significant interaction of IgA and number of adults indicates a moderating interaction between these terms on the number of 4th-stage larvae, as both increase.)

Variable	Effect	Standard error	Probability
Intercept	2·56	0·07	0·001
Number of adults	1·23	0·13	0·001
IgA * number of adults	1·89	0·52	0·001

stituted for log-transformed number of *T. circumcincta* in the multiple regression model, it was significant ($P < 0{\cdot}001$) but when year and the log-transformed number of *T. circumcincta* were included in the same model, neither was significant ($P > 0{\cdot}30$).

DISCUSSION

This study has measured plasma IgA activity against 4th-stage larvae of *T. circumcincta* using a simple, indirect ELISA. IgA activity in different sheep was positively skewed with most lambs having relatively low activity. A Box-Cox procedure was used to determine a suitable transformation; subsequent analyses on transformed data found that IgA activity was repeatable and heritable. Variation among animals in IgA activity was associated with differences in month of sampling, year, sex, sire and dam. Animals with increased IgA activity had lower egg counts and shorter adult female nematodes. In some but not all years, lambs with more IgA also subsequently acquired more *T. circumcincta*.

The distribution of IgA activity among lambs was positively skewed. Sinski *et al.* (1995) detected IgA activity against *T. circumcincta* in the abomasal mucosa and showed that IgA activity in the serum was dependent upon the abomasal activity and the number of adult nematodes present in the abomasum. Following deliberate infection, the distribution among sheep of IgA activity in the abomasum was not significantly different from the normal distribution but the distribution of *T. circumcincta* among hosts was highly skewed (Stear *et al.* 1995*a*). Therefore the observed skew of IgA activity in the plasma may reflect the skewed distribution of nematodes among hosts. In ruminants, serum IgA is dimeric and in experimental studies almost totally derived from the intestine (Sheldrake *et al.* 1984). Together these results suggest that plasma IgA activity can provide a window on local IgA responses in sheep.

Sire, dam, year, sex and month of sampling were all associated with variation in IgA activity. There was a peak in IgA activity against *T. circumcincta* in September. The subsequent decrease in IgA activity may be a consequence of the decline in the number of infective larvae on pasture at this time (Urquhart *et al.* 1987). The association with sex was due to higher IgA activity in female lambs than their castrated male contemporaries. This difference may account, at least in part, for the tendency of females to have lower faecal egg counts than castrated males (Barger, 1993; Stear *et al.* 1995*a*).

The importance of sire and dam was indicated by the relatively high heritability estimate of 0·56. This relatively high value may even underestimate the heritability of abomasal IgA responses because the number of *T. circumcincta* in the abomasum influences the transfer of IgA to the plasma. In lambs the heritability of the number of *T. circumcincta* is very low and may even be zero (Stear *et al.* 1997). Consequently, variation in the number of *T. circumcincta* will act as a source of non-genetic noise on plasma IgA concentrations. This noise may reduce the heritability estimate for plasma IgA below that of abomasal IgA. Our high heritability estimate for IgA activity is consistent with the hypothesis that IgA regulates worm growth and fecundity and with our previous findings of high estimated heritabilities for worm length and worm fecundity (Stear *et al.* 1997).

A high heritability for abomasal IgA is plausible. Hatagima, Cabello & Krieger (1999) used path analysis to estimate the heritability of IgA level at $0{\cdot}41 \pm 0{\cdot}03$ in people from Brazil. As stated by the authors, their procedure will unavoidably lead to underestimates of the true heritability. In addition, the heritability of activity against a single parasite is likely to be higher than the heritability of total IgA levels so long as there is genetic variation in specific IgA responses. Further, potential quantitative trait loci influencing IgA levels in humans have been found on chromosomes 10 and 13 (Wiltshire *et al.* 1998). In contrast, in cattle, Gasbarre, Leighton & Davies (1993) did not find any evidence for genetic variation in IgA responses to *Ostertagia ostertagi* while Mallard *et al.* (1983) did not find convincing evidence for genetic variation in serum IgA concentrations.

There was an association between IgA activity and the number of adult *T. circumcincta* acquired over the following 6–7 weeks, but this association was present in only 2 of the 4 years studied. Previous studies in deliberately infected sheep (Stear *et al.* 1995*b*) have not found an association between IgA activity and worm number. Therefore, the association between IgA activity and worm number is, at best, inconsistent and cannot be used to indicate worm numbers.

The results of this study confirm the previous association between increased IgA activity and reduced mean length of adult female *T. circumcincta* (Stear *et al.* 1995*b*) as well as the association between

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the number of adult nematodes and adult female worm length (Stear *et al*. 1995*b*). The mechanisms responsible for the immune and density-dependent effects have still to be described at the molecular level. At this stage we do not know whether these effects operate independently or together in regulating worm length.

A detailed study of the development of immunoglobulin concentrations in lambs and ewes has been provided by Klobasa & Werhahn (1989*a*, *b*). They found IgA in the plasma of ewes and sheep was present at low levels relative to IgG1, IgG2 and IgM. Nevertheless, our study has shown that increased IgA activity was associated with decreased faecal egg counts, decreased worm length and an increased number of 4th-stage larvae. As IgA is heritable and associated with resistance to deliberate infection (Smith *et al*. 1985; Stear *et al*. 1995*a*) as well as natural infection (this study), it appears to be a possible marker of resistance to infection.

The importance of IgA in parasitic infections has recently been reviewed by Wedrychowicz (1995). In particular, IgA may play a role in the eosinophil-mediated killing of schistosomes (Dunne *et al*. 1993), may also be important in *Trichuris muris* infections of mice (Roach *et al*. 1991) and intestinal IgA from infected mice and rats decreases the fecundity of *Trichinella spiralis* (Jacqueline *et al*. 1978). In addition, Gill *et al*. (1993) have reported an association between increased IgA and IgG1 in serum and faeces and decreased egg counts following infection with *Haemonchus contortus*. More recently, Claerebout & Vercruysse (2000) have reported an association in cattle between increased abomasal IgA responses and reduced egg production by *Ostertagia ostertagi*.

In conclusion, the associations between increased IgA activity and reduced faecal egg count, reduced adult female worm length and increased numbers of 4th-stage larvae found following deliberate infections have been confirmed following natural infections. In addition, IgA activity against *T. circumcincta* shows promise as an indicator of resistance to infection in lambs.

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