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Salivary IgA: a biomarker for resistance to *Teladorsagia circumcincta* and a new Estimated Breeding Value

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Abstract

Teladorsagia circumcincta is the dominant nematode of sheep in cool, temperate climates. Faecal nematode egg counts (FEC) are widely used to identify the intensity of infection and as a measure of host resistance to nematodes. However due to density-dependent effects on worm fecundity the relationship between FEC and worm burden is not linear. In addition collecting FEC data is challenging on a practical level and there is a need for more reliable markers of resistance. There are two major known mechanisms of immunity to *T. circumcincta*: IgE against third stage larvae (L3), which inhibits larval establishment and IgA against fourth stage larvae (L4), which inhibits parasite growth. We measured salivary IgA responses in over 5000 animals against L3 antigen by Enzyme Linked Immunosorbent Assay (ELISA). Antigen-specific IgA levels were negatively correlated with FEC (r=-0.26, SE=0.02) and were heritable (h^2 = 0.16, SE=0.04) indicating that they can be used to identify resistant animals suitable for inclusion in selective breeding programs. Ecological theory predicts that a trade-off between immunity and host-growth will exist due to competing energetic needs. Antigen-specific IgA

responses were not negatively correlated with muscle deposition such that the expected trade-off between growth and immunity was not apparent. Our analyses indicate that selection for antigen-specific IgA is possible without impacting on the production traits for the Lleyn breed.

1. Introduction

Infection with gastro-intestinal strongyles such as *Teladorsagia circumcincta* presents a significant problem to the sheep breeding industry, impacting both the welfare and productivity of animals. Anthelminthics are commonly used to control helminth infection but reliance on this method alone is ill-considered in light of growing anthelminthic resistance (Traversa and von Samson-Himmelstjerna, 2016). An integrated approach to ensure sustainable control strategies for the future is the main objective of research in this area and requires improved phenotypic and genetic markers of resistance and resilience to inform selective breeding programs (Jackson et al., 2009).

Whilst both resistance and resilience contribute to control of helminth infection it may be more preferable when considering productivity to select for resilient sheep that can better tolerate the infection and grow in spite of worm

burden. Previous research has demonstrated that there are two main mechanisms of immunity to *T. circumcincta* infection. Immunoglobulin E (IgE) against third stage larvae (L3), which inhibits larval establishment and Immunoglobulin A (IgA) against fourth stage larvae (L4), which inhibits parasite growth (Lee et al., 2011; Martinez-Valladares et al., 2005; Murphy et al., 2010; Strain et al., 2002). The IgE response, although protective is associated with pathology that leads to a relative protein deficiency in the animal (Stear et al., 2003). Thus resistance to the infection is costly both in terms of welfare and productivity. The IgA response impairs the growth of the worms resulting in shorter and crucially less fecund worms that contribute less to pasture contamination (Stear et al., 1995; Strain et al., 2002). IgA without the pathology and negative impact on animal growth associated with IgE has potential as a marker of resilience.

Traditionally low faecal nematode-egg count (FEC) is used as a marker of resistance in selective breeding programmes. However, mathematical modelling indicates that IgA has advantages over FEC as a selection tool; after 7 generations of selection based on plasma IgA a drop in FEC of 85% was achieved whereas selection on FEC alone gave only a 50% reduction (Prada Jimenez de Cisneros et al., 2014). There

are also other disadvantages of using FEC as a measure of infection intensity; worm density-dependent effects on egg production mean that high worm burdens do not always tally with high FEC (Bishop and Stear, 2000; Romeo et al., 2014; Smith et al., 1987), sampling per rectum is invasive and often samples can't be obtained. Measuring IgA in plasma is also invasive and requires specialist training.

Salivary IgA to a carbohydrate larval antigen (CARLA) has been used successfully to measure immunity to gastrointestinal nematodes (Shaw et al., 2012). Here we have developed an Enzyme Linked Immunosorbent Assay (ELISA) to measure *T. circumcincta*-specific IgA in saliva. The test measures the IgA response to L3 larvae with the rationale that resistance results from the recognition of multiple antigens (Ellis et al., 2014). This test is deliberately targeted to the predominant nematode infection in the UK and other temperate climates. Breeders and veterinarians have readily adopted this test and results are reported as an Estimated Breeding Value (EBV) for inclusion in the selective breeding programmes of the Lleyn Performance Recording group.

2. Material & Methods

2.1 *Animals and sample collection*

Eighteen farms owned by Performance Recorded Lleyn Breed Society members throughout the UK were involved in the study and bred all the Lleyn animals that were tested (n=5201). Sampling was undertaken between the months of July-September to coincide with exposure to *Teladorsagia circumcincta* through grazing. For lambs sampled between 2014 and 2017 the average age at sampling was 193days of age. The age distribution of animals in the study is shown in Figure 1.

Saliva samples were collected by insertion of a dental swab (Robinson Healthcare, 12mm), secured by forceps, into the cheek pouch of the animal. Swabs were sealed in 15ml falcon tubes and saliva recovered by centrifugation at 449 rcf for 5 minutes. Saliva was then frozen at -20^{o}C before use in ELISA.

2.2 *Antigen and ELISA*

T. circumcincta antigen was prepared by homogenisation of 1 million L3 in 10mM Tris-HCL with addition of protease inhibitors (0.5M EDTA, 0.5M EGTA, 1M NEM, 0.33M PMSF, 0.1M TPCK, 1mM pepstatin) and 2% sodium deoxycholate. Homogenisation was performed at 30Hz for 6 mins in a Retsch MM400 mixer mill. Antigen was passed through a 0.2µm syringe filter and frozen before use in ELISA.

158

159 Antigen-specific salivary IgA ELISA: 96 well BD Falcon
160 ELISA plates were coated with 5µg/ml L3 antigen diluted in
161 0.06M carbonate buffer (0·04 M NaHCO3, 0·02 M NaCO3,
162 pH9·6) and incubated overnight at 4°C. Non-specific
163 binding was blocked with 4% Marvel milk powder in
164 Phosphate-buffered saline with 0·1% Tween 20 (PBST) for
165 2 h at 37 °C. Wells were washed three times in PBST after
166 each subsequent step. Saliva samples were added at 1:4
167 dilution using PBST as a diluent and incubated for 30
168 minutes at 37 °C. Isotype-specific detection antibody
169 horseradish peroxidase conjugated rabbit anti-ovine IgA
170 (AbD serotec AHP949P) was added at 1:15 000. Plates were
171 incubated for 30 minutes at 37 °C. An additional wash in
172 distilled water was carried out before developing with 3,3',
173 5,5'-tetramethylbenzidine (TMB) peroxide substrate
174 (Pierce ™), at room temperature for 5 min. Optical density
175 (O.D.) was read at 450nM using a spectrophotometer.
176 Results were expressed as an O.D. Index calculated as
177 (sample O.D. – control O.D.)/ (high-responder O.D. – control
178 O.D.). This O.D. Index and animal pedigree were used to
179 generate an estimated breeding value for antigen-specific
180 IgA (IgA EBV).

181

182 To ensure standardisation of IgA responses from year to
183 year the high responder pool was created from 6-10 sheep
184 and this provides enough material to test thousands of
185 animals. When a new batch of high responder pool is
186 created the reactivity of the previous pool and the new pool
187 are carefully compared by testing multiple samples with
188 both batches.
189
190 2.3 *Calculation of IgA Estimated Breeding Value (EBV)*
191 At the time of producing IgA EBVs, raw antigen-specific IgA
192 data were available for 5,201 Lleyn animals, measured
193 between 2014 and 2016 from 15 flocks. Basic data edits
194 were undertaken to remove data for animals that were
195 sampled multiple times or outside of 100 to 350 days of
196 age. To normalise the IgA phenotype distribution a Box-Cox
197 procedure was applied using a square root transformation
198 and extreme outliers removed. To allow genetic parameter
199 estimation with the other traits routinely evaluated in the
200 national Lleyn genetic evaluation, Faecal Egg Count (FEC)
201 (log transformed), birth weight (kg), 8 week weight (kg), 21
202 week weight (kg), ultrasound muscle depth (mm),
203 ultrasound fat depth (mm) and ewe weight at tupping (kg)
204 were extracted from the genetic evaluation database for the
205 flocks that recorded antigen-specific IgA. For genetic
206 parameter estimation, animals that were fostered, the

result of embryo transfer, not purebred or from a litter size greater than 3 were removed from the dataset. Any record which was part of a small (< 5 animals) or single sire contemporary group were set to missing, as were records outside 3 standard deviations from the contemporary group mean.

Contemporary groups (CG) were as defined in the routine national genetic evaluations (Ceyhan, 2015). For birth- and 8 week- weight the CG was defined as flock of birth, season and sex (Flock-Season-Sex CG). The same definition, but with the inclusion of a user defined management group (Flock-Season-Sex-Managementgroup CG) was used for 21 week weight, ultrasound muscle and fat depth and Faecal Egg Count. In all cases, season was based on date of birth and within year (defined from August to July) was sliced so that consecutive animals were not more than 28 days apart in age and the total time span of a CG was not more than 155 days (5.5 months). The CG for ewe weight at tupping was the herd and year of tupping (Tupping herd-year CG). For IgA it was decided to use a similar CG as for 21 week weight, ultrasound scans and FEC, however including the IgA management group. A 5 generation pedigree was built for animals with phenotype information.

For both parameter estimation and the calculation of EBVs the statistical models used for routine genetic evaluation were used for all traits other than IgA. The statistical models fitted were as follows;

Birth weight = Flock-Season-Sex CG + litter size born + dam age + animal genetic effect + dam genetic effect + permanent environment effect

8 week weight = Flock-Season-Sex CG + litter size reared + dam age + animal genetic effect + dam genetic effect + permanent environment effect

21 week weight / ultrasound muscle depth /ultrasound fat depth / FEC = Flock-Season-Sex-Managementgroup CG + litter size reared + dam age + scanning age + Flock-Season-Sex-Managementgroup CG * scanning age + animal genetic effect

Ewe weight at tupping = Tupping herd-year CG + animal genetic effect

The CG, litter size and dam age terms were fitted as fixed class effects. CG was as defined above, litter size born was the number of lambs born in the same litter, litter size reared was the number of lambs born and raised in the same litter. Dam age was the age of the dam in years, with dams over 5 years of age being recorded as 5 yrs.

Scanning age was the age of the animal at the time of the 21 week weight and ultrasound scans were measured. It is recorded in days and was fitted in the model as a fixed covariate.

Fitted as random effects were the additive, dam and permanent environment genetic effects.

The final statistical model for IgA was as follows;

IgA = Flock-Season-Sex-ManagementGroup CG + litter size born + age when IgA sample taken + animal genetic effect

Model terms were fitted in the same manner as above, with age at IgA sample being recorded in days and fitted as a fixed covariate.

ASReml (Gilmour, 2009) was used to estimate genetic parameters to produce EBVs. To estimate (co)-variance components, a series of uni- and bi- variate animal models (described above) were fitted. The software MiX99 (Lidauer, 2011) was used to produce EBVs based on the variance components estimated and the models described above.

3. Results

After basic data edits antigen-specific IgA records were available for 5,188 animals, of which 4,473 also had FEC records available in the national Lleyn genetic evaluation. Table 1 describes the raw data that was available for genetic parameter estimation. A small to moderate heritability of 0.16 (0.04) was estimated for antigen-specific IgA transformed to a normal scale (Table 2), this was double that of the heritability calculated for FEC in the same cohort (Table 2). In addition to being heritable, there was sufficient genetic variation to enable selection of animals for increased antigen-specific IgA activity.

Genetic relationships estimated from bi-variate analysis are reported in Table 2. Moderate genetic correlations between IgA and FEC were estimated with a negative correlation (-0.26 (0.02)) between IgA and FEC Strongyles. These correlations indicate that as IgA increases genetically, FEC Strongyles decreases improving host resistance for these types of worms. A positive correlation (0.27 (0.19)) between IgA and FEC Nematodirus was also observed, however this correlation estimate was not significantly different from 0.

Parameters were unable to be estimated for birth and eight week weight due to convergence issues. The correlations between antigen-specific IgA activity and production traits were not significantly different from 0. These results indicate that selection for antigen-specific IgA is possible without any adverse influence on production traits for the Lleyn breed.

Based on the genetic parameters estimate, EBVs were produced for IgA with the only correlation fitted for IgA being with FEC Strongyles (-0.26 (0.02)). The average IgA EBV was 0.00 and ranged between -0.17 and 0.18 with a standard deviation of 0.01. A simple correlation between sire EBV and progeny IgA was undertaken for sires with more than 10 progeny recorded for IgA. After adjusting the phenotypes for the fixed effects fitted in the statistical model, a correlation of 0.91 was observed (Figure 2). This relationship suggests that sires with higher EBVs for IgA will produce offspring with genetics to produce higher IgA responses.

4. Discussion

This study has shown that antigen-specific salivary IgA responses are heritable and negatively correlated with FEC making them suitable to identify animals resistant to

nematodes for use in selective breeding programs. Antigen-specific IgA responses have twice the heritability of FEC (which is currently used to identify resistant animals) suggesting that selection based on IgA would be more efficient. Furthermore we predict there would be no adverse effect of selection on production traits (correlations between antigen-specific IgA and fat and muscle deposition were not significantly different from zero). This is in contrast to ecological theory which predicts that a trade-off between immunity and host-growth will exist due to competing energetic needs (Klasing, 2004). It is possible that this trade-off is not evident for the measures of immunity and growth used here because the IgA response to *T. circumcincta* is not known to cause pathology and a relative protein deficiency as is the case for the IgE response (Stear et al., 2003).

Previous research has focussed on plasma IgA responses to *T. circumcincta* L4 antigens (JP et al., 2014; Strain et al., 2002). Here we opted to use L3 antigens as they are easier and less costly to produce than L4 sourced antigens. There is good evidence that L3 antigens can be used as a proxy for L4 (McRae et al., 2014) and a strong correlation between plasma IgA responses to L3 and L4 antigens has previously been shown (Stear et al., 1995). Despite being easier to

356 produce it is likely that responses to L3 antigens will have
357 weaker relationships to FEC and perhaps productivity than
358 L4 and in the future the use of L4 antigens or a suite of L4
359 recombinant antigens in ELISA may be advantageous.
360 Indeed the heritability of IgA activity to L4 antigens in
361 plasma of Scottish Blackface sheep (0.56 ± 0.11) (Strain et
362 al., 2002) was higher than that measured here to L3
363 antigens in saliva of Lleyn sheep. It should be noted
364 however that these two heritabilities are not directly
365 comparable as different data transformations and statistical
366 models were used. Importantly in each case the heritability
367 of IgA responses was approximately twice that of FEC
368 (Bishop, 1996).
369
370 The strength of the relationship between salivary IgA and
371 plasma IgA or indeed mucosal IgA at the site of infection
372 (abomasum) remains to be determined. However, the
373 majority of IgA present in saliva is derived from B-
374 lymphocytes that migrate from the gut associated lymphoid
375 tissue (Brandtzaeg, 2007a, b). This underlying biology and
376 previous use of L3 antigens in place of L4 (McRae et al.,
377 2014) supports our use of salivary IgA responses to L3
378 antigens as an indicator of the protective mucosal IgA
379 response to L4 antigens. This rationale was upheld by the
380 favourable genetic correlation with FEC that we observed.

We advocate using salivary IgA as an indicator of resistance to *T. circumcincta* as on practical level saliva is easy to collect for farmers or veterinarians and there is no possibility of animals not providing a sample. Samples are also easy to process in large numbers and, as salivary IgA is quite stable, can be shipped at room temperature. Alternatively the saliva (or saliva soaked swab) can be stored at -20^{o}C prior to analysis without impacting the result.

Selection for resistance to nematodes based on FEC in the UK requires an average flock level of 100 eggs per gram (epg) or higher (Signet Breeding Services, 2014). For FEC it is also crucial that lambs have not been treated with anthelmintics for at least 4 weeks. IgA activity is less affected by anthelmintic use and (as with FEC) flock EBV will be useful for animals that have been exposed to similar levels of infection by grazing the same pasture. Currently the Lleyn breeders are utilising both FEC and IgA EBVs, as the required flock responses in IgA activity are still to be determined.

In this study we measured antigen-specific responses to *T. circumcincta* larval antigens. Of course the animals were

406 grazed on pasture and so would be exposed to and infected
407 with other parasitic nematodes (e.g. *Nematodirus battus*,
408 *Cooperia curticei*, *Oesophagostumum* and *Trichostronglyus*
409 species). We would anticipate some cross-reactivity in the
410 antibody response to *T. circumcincta* and these other
411 species and this may be beneficial to the host. However,
412 larval culture for speciation performed on a subset of faecal
413 samples revealed these lambs to be predominantly infected
414 with *T. circumcincta* (unpublished). In addition to the
415 favourable negative correlation between IgA and FEC
416 Strongyles, a positive correlation between IgA and FEC
417 Nematodirus was observed (Table 2). However, this
418 correlation estimate was not significantly different from 0.
419 The difference in direction was unexpected, given that a
420 strong positive genetic correlation (0.61 (0.16)) between
421 the two FEC traits was estimated. It is worth noting that
422 Nematodirus counts are zero-inflated (Denwood et al.,
423 2008) and so estimates using faecal egg counts for this
424 species need to be treated cautiously.
425
426 5. Conclusions
427 Salivary IgA responses to *T. circumcincta* larval antigens
428 can be used to identify animals resistant to nematodes for
429 inclusion in selective breeding programs. Antigen-specific
430 IgA responses have twice the heritability of FEC (which is

currently used to identify resistant animals) suggesting that selection based on IgA would be more efficient. On a practical level saliva is easy to collect for farmers or veterinarians and there is no possibility of animals not providing a sample. The research reported here was conducted on Lleyn sheep, however the IgA test is appropriate for use throughout the sheep breeding industry as a tool for breeders to identify animals resistant to nematode infection. Selection of animals based on the IgA test could play an important role in the integrated management of *T. circumcincta* and reduce the need for treatment with anthelmintics.

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565

566

567 Table 1: A summary of the edited Lleyn data used to

568 estimate genetic parameters

Trait	n	average	min	max	Std. dev
birth weight (kg)	66,626	4.14	2.0	7.0	0.96
8 week weight (kg)	268,594	19.17	5.7	32.8	4.36
21 week weight (kg)	102,035	35.76	13.0	58.6	7.44
Ultrasound muscle depth (mm)	63,499	24.08	13.7	34.5	3.41
Ultrasound fat depth (mm)	62,825	2.60	0.1	7.13	1.36
ewe weight at tupping (kg)	13,398	54.71	20	90	12.12
Faecal egg count – Strongyles (log transformed)	5,840	5.12	0.0001	9.00	2.08
Faecal egg count – Nematodirus (log transformed)	5,840	1.89	0.0001	7.62	2.36
transformed IgA	4,622	0.71	0.03	1.99	0.35

569

570

571 Table 2: Estimates of phenotypic variances, heritability and

572 phenotypic and genetic correlations between transformed

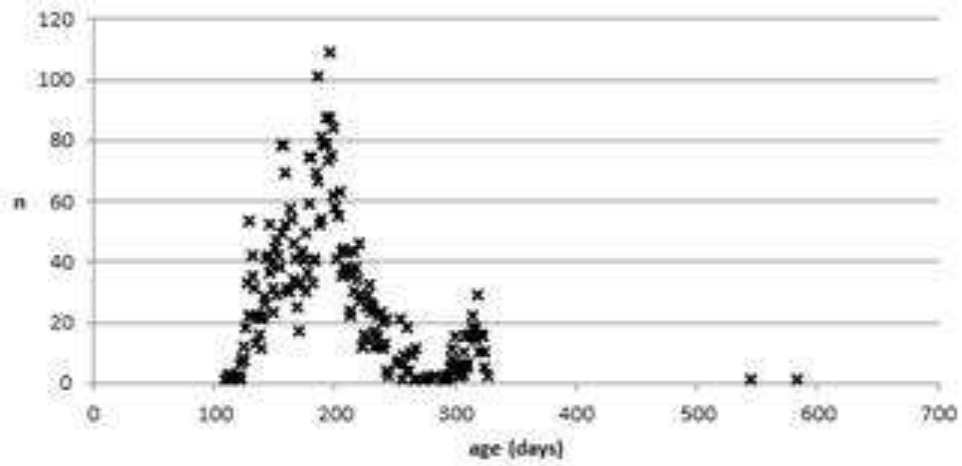
573 IgA and production traits of UK Lleyn sheep

Trait	**Phenotypic Variance**	**Heritability**	**Phenotypic correlation with IgA**	**Genetic correlation with IgA**
transformed IgA	0.08 (0.002)	0.16 (0.04)	-	-
Faecal egg count – Strongyles (log transformed)	2.68 (0.06)	0.08 (0.03)	-0.002 (0.02)	-0.26 (0.02)
Faecal egg count – Nematodirus (log transformed)	4.55 (0.11)	0.15 (0.04)	0.007 (0.02)	0.27 (0.19)
Ultrasound muscle depth (mm)	4.85 (0.09)	0.37 (0.03)	0.05 (0.02)	0.08 (0.12)
Ultrasound fat depth (mm)	1.04 (0.02)	0.41 (0.03)	0.04 (0.02)	0.04 (0.12)
21 week weight (kg)	20.54 (0.25)	0.45 (0.02)	0.02 (0.02)	-0.02 (0.11)
ewe weight at tupping (kg)	37.81 (0.85)	0.37 (0.03)	0.04 (0.04)	0.25 (0.15)

574

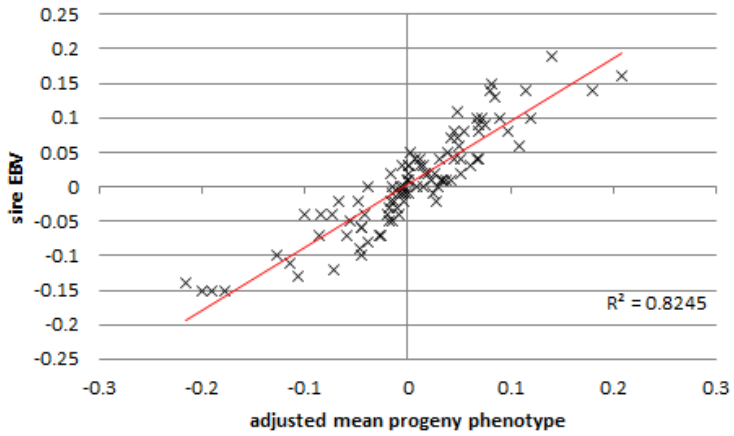
575

Figure 1: Age distribution of animals sampled between 2014 and 2017. Animals greater than 350 days old were excluded from final analysis. The mean age at sampling for all animals was 193 days. For males the mean age was 182 days and for females 196.



584 Figure 2: Sire IgA EBV and the mean progeny IgA phenotype

585 adjusted for fixed effects



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