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Research paper

Quantifying the sources of variation in eosinophilia among Scottish blackface lambs with mixed, predominantly *Teladorsagia circumcincta* nematode infection

Caitlin J. Jenvey [a], Fazel Almasi [a], Emma U. Halliwell [a], Xia Li [b], David Piedrafita [c], Sarah Preston [c], Michael J. Stear [a,*]

[a] AgriBio Centre for AgriBioscience, Department of Animal, Plant and Soil Sciences, La Trobe University, Bundoora, Victoria, 3086, Australia
[b] Statistical Consulting Platform, La Trobe University, Bundoora, Victoria, 3086, Australia
[c] School of Science, Psychology and Sport, Federation University Australia, Victoria, 3353, Australia

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ABSTRACT

Eosinophils play a key role in defence against gastrointestinal nematodes. There is considerable variation among animals in the intensity of eosinophilia following nematode infection. However, the statistical distribution of eosinophils among animals has still to be determined. A better description of the variation among animals could provide biological insight and determine the most appropriate way to analyse the effect of eosinophils. We estimated blood eosinophil numbers in a flock of Scottish Blackface sheep that were naturally exposed to mixed, predominantly *Teladorsagia circumcincta* infection. Three of the four eosinophil counts were better described by a gamma distribution than by a lognormal distribution. The scale and shape parameters of the gamma distribution varied over time. Eosinophil counts differed among animals kept on separate fields before weaning and between singletons and twins but were not significantly different between years and genders. Eosinophil counts also differed among offspring from different sires and dams. The parameters of the gamma distribution were used to enable a power analysis. Large numbers of animals were required to reliably detect even large differences between two groups. These results indicate that methods appropriate for gamma distributions, such as generalized linear mixed models, will provide more reliable inferences than traditional methods of analysis and experimental design.

1. Introduction

Eosinophilia is the hallmark of all parasitic infections. In sheep, eosinophils have been shown to be involved in host responses to gastrointestinal nematodes (GIN). In response to *Haemonchus contortus*, eosinophils have been shown to kill larvae *in vitro* (Rainbird et al., 1998; Terefe et al., 2007, 2009), while in response to *Teladorsagia circumcincta*, eosinophils appear to mediate protective immunity (Stevenson et al., 1994), being strongly associated with mucosal IgA activity against L4, and reduced fecundity and worm length (Stear et al., 1995a, 2002). Eosinophils have also been used to identify resistant animals (Hohenhaus et al., 1998) although their value is disputed (Stear et al., 2002; Woolaston et al., 1996).

Despite their importance, little is known about the factors that influence eosinophil numbers in ruminant responses to GIN. There is considerable variation among lambs in the number of eosinophils recruited in response to nematode infection and lambs with higher blood and tissue eosinophil counts are more resistant to infection (Stear et al., 1995a). However, the statistical distribution of the variation among animals has not been identified, although it is clearly not a normal distribution (Stear et al., 1995a, 2002). The distribution is of biological interest because it is important for disease transmission and the impact of infection on flocks. The distribution also determines the most appropriate method of data analysis (McCullagh and Nelder, 1989). Analysing data with a statistical procedure that does not appropriately account for the distribution of the data is unlikely to give accurate probabilities and could lead to incorrect inferences. Therefore, this study aimed to determine the distribution of eosinophils, develop appropriate methods of analysis, identify the sources of variation and finally, carry out a power analysis to aid the design of powerful experiments.

* Corresponding author.
E-mail address: M.Stear@latrobe.edu.au (M.J. Stear).

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2. Materials and methods

2.1. Animals and data collection

The samples used for this study were collected in August and September of 1993 and 1994 from 400 Scottish Blackface lambs on a commercial, upland farm in Southwest Strathclyde, Scotland. Lambs consisted of ewe lambs and castrated male lambs, naturally exposed to predominantly *Teladorsagia circumcincta* nematode infection, and the procedures used have been previously described in Stear et al. (2002). Animal information, including gender (male, female), field (1, 2, 3), birth type (single, twin), age, sire and dam were also collected. Field refers to the field that the lambs were kept before weaning at 4 months of age. After weaning all lambs were kept on the same field. Blood samples were collected and the concentration of eosinophils in peripheral blood was estimated by adding 10 µl of whole blood to 90 µl of Carpentier's solution (Dawkins et al., 1989), left for at least 5 min and duplicate samples were counted using a haemocytometer. Each cell counted represented 5.6 cells / µl of blood.

2.2. Statistical analysis

The parameters of the gamma and lognormal distributions were obtained with the Univariate procedure of SAS on Demand for Academics. The Univariate procedure also provided empirical distribution tests (Kolmogorov-Smirnov, Cramer-Von Mises and Anderson-Darling) to compare the fits of the two distributions to the eosinophil counts. The parameters of the distributions were estimated by maximum likelihood. For gamma distributions when the shape parameter was less than one, the shape parameter was specified to produce probability values for the empirical distribution function tests. Generalized linear mixed modelling (McCullagh and Nelder, 1989) was performed with the Glimmix procedure. The generalized linear model relates the non-normal response variable to the linear model by a link function (Nelder and Wedderburn, 1972). A gamma distribution was used along with the log link and each month was fitted separately. Age in days was fitted as a covariate while the fixed effects were gender (female or castrated male), field (1, 2 or 3), year (1993 or 1994) and birth type (single or twin). The random effects were sire and dam. There were 24 sires and 242 dams.

The power analysis was performed in R version 4.0.4 (R Core Team, 2020) and used source code written by Cundill and Alexander (2015). The function 'expand.grid' was used to determine sample size at power values ranging from 0.02 to 0.98. Alpha was set to 0.05, Q0 was set to 0.5 (equal arms; balanced design) and method was set to 2 (mu1 is used for the intervention arm under the null hypothesis). The power analysis calculated the total number of animals required to achieve a given power for a given difference between two groups. Each power analysis was plotted using the function 'geom_line' in the package 'ggplot2' (Wickham, 2016).

In order to highlight the variation in the probability density function for the Gamma distribution, 300,000 data points from a gamma distribution with the specified shape and scale parameters were sampled in SAS and plotted using the Sgplot program.

3. Results

3.1. The distribution of eosinophil counts

The number of eosinophils showed considerable variation among animals. Most animals had relatively low counts but a small proportion of lambs had quite high counts (Fig. 1). Mean eosinophil counts varied from 67 cells per ul in the samples from September 1993 to 87 cells per ul in September 1994 (Table 1). The standard deviation ranged from

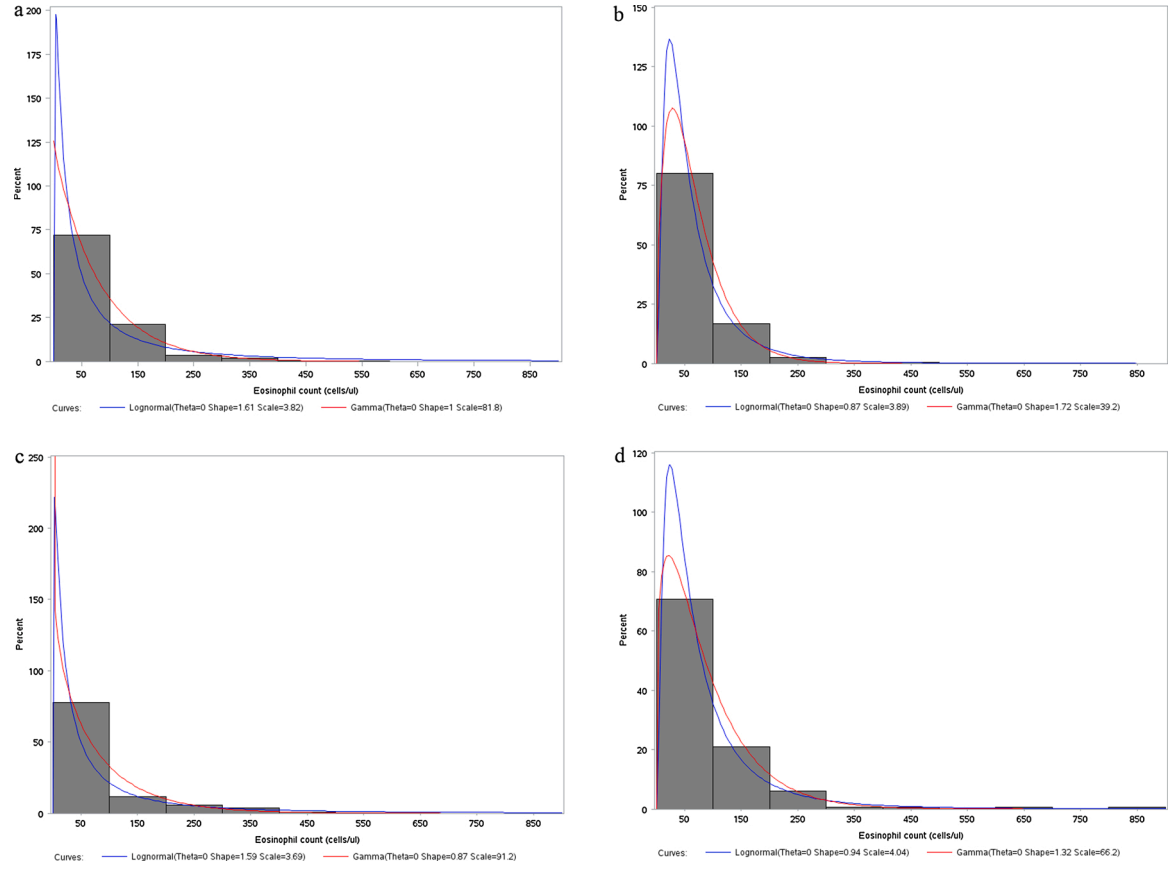

Fig. 1. The distribution of eosinophil concentrations among sheep in August.

Table 1
Gamma distribution parameters for eosinophil counts from Scottish Blackface lambs.

Month	Year	Shape	Scale	Mean	Standard deviation
August	1993	0.99	81.6	81.6	81.7
September	1993	1.72	39.2	67.4	51.4
August	1994	0.87	91.2	79.0	84.9
September	1994	1.32	66.2	87.2	75.9

51.4 in samples collected in September 1993 to 84.9 in August 1994 (Table 1). Eosinophil counts were visually more similar to a gamma distribution than to a lognormal distribution. Three of the four distributions showed that the gamma distribution was a better fit than the lognormal. The visual impression was supported by empirical distribution tests (Table 2). The exception was the eosinophil counts in September 1994 where the gamma distribution was closer to the observed distribution at low counts but underestimated the number of animals with extremely high counts. However, the deviation from the gamma distribution was largely due to three extremely high observations. When these three were discarded, the truncated distribution was closer to a gamma distribution with p-values of 0.076, 0.023 and 0.013 for the Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darling tests, respectively.

3.2. Generalized linear mixed models

The analysis showed that in August, the fixed effects of preweaning field ($p = 0.0395$) and birth type ($p = 0.0158$) were significant, but not the effects of year ($p = 0.737$) or age ($p = 0.345$). The variance components for sire, dam and the residual were 0.10 ± 0.05, 0.25 ± 0.08 and 0.54 ± 0.07. Likelihood ratio tests based on the residual pseudo-likelihood in independent models indicated that both sire ($p < 0.001$) and dam ($p < 0.001$) were significant.

In September, the analysis failed to converge but did converge when the effect of dam was excluded from the analysis. The effects of year ($p = 0.342$), field ($p = 0.061$), birthtype ($p = 0.1133$) and age ($p = 0.936$) were not significant. The estimated variance for sire was 0.09 ± 0.04 while the residual variance was 0.67 ± 0.05.

Table 2
Empirical distribution function tests of the fit of the gamma and lognormal distributions to eosinophil counts.

Date	Distribution	Test	P value
August 1993	Lognormal	Kolmogorov-Smirnov	<0.010
August 1993	Lognormal	Cramer-von Mises	<0.005
August 1993	Lognormal	Anderson-Darling	<0.005
August 1993	Gamma	Kolmogorov-Smirnov	0.193
August 1993	Gamma	Cramer-von Mises	> 0.250
August 1993	Gamma	Anderson-Darling	0.180
September 1993	Lognormal	Kolmogorov-Smirnov	0.018
September 1993	Lognormal	Cramer-von Mises	<0.005
September 1993	Lognormal	Anderson-Darling	<0.005
September 1993	Gamma	Kolmogorov-Smirnov	>0.500
September 1993	Gamma	Cramer-von Mises	>0.500
September 1993	Gamma	Anderson-Darling	>0.500
August 1994	Lognormal	Kolmogorov-Smirnov	<0.010
August 1994	Lognormal	Cramer-von Mises	<0.005
August 1994	Lognormal	Anderson-Darling	<0.005
August 1994	Gamma	Kolmogorov-Smirnov	0.191
August 1994	Gamma	Cramer-von Mises	0.248
August 1994	Gamma	Anderson-Darling	0.206
September 1994	Lognormal	Kolmogorov-Smirnov	>0.150
September 1994	Lognormal	Cramer-von Mises	0.496
September 1994	Lognormal	Anderson-Darling	0.380
September 1994	Gamma	Kolmogorov-Smirnov	<0.001
September 1994	Gamma	Cramer-von Mises	<0.001
September 1994	Gamma	Anderson-Darling	<0.001

3.3. Power analysis of eosinophil counts between genders

The power analyses for eosinophil counts in August and September are presented in Fig. 2. The power analysis indicates the probability of detecting real differences between groups of different sizes.It assumes that there are no other effects in the statistical model or that these effects have already been accounted for. If one group has twice as many eosinophils as another group then there is a 95 % chance of finding a statistically significant difference between the two groups if there are 50 animals in each group. Smaller numbers in each group would decrease the chances of finding a significant difference between the groups. Similarly, if one group had three times as many eosinophils, a total of about 40 animals equally distributed between the two groups would be required. Where one group had 4 times the eosinophil count in the other group about 15 animals in each group would be required.

4. Discussion

Eosinophil counts from 400 Scottish Blackface lambs were better described by a Gamma distribution than by a lognormal distribution. A generalized linear mixed model with a Gamma link function determined the most important animal factors that influenced eosinophil numbers. The results from the generalized linear mixed models were used to enable a power analysis. This analysis demonstrated that large numbers of animals would be required to detect large differences between groups of animals.

Eosinophil counts were positively skewed and most animals had relatively low numbers of blood eosinophils but a small proportion had quite high counts. Immunological and evolutionary theory predicts that immune responses to important endemic diseases are close to optimal for most animals; this is a consequence of the fundamental theorem of natural selection (Fisher, 1930). Therefore, the relatively low responses of most animals are somewhat surprising. More research is required to explain this finding. The skewed eosinophil response of animals was better described by a gamma distribution than by a lognormal distribution. Therefore, analytical methods that employ a gamma distribution, such as generalized linear models, are more likely to provide correct inferences than the traditional log transformation.

The gamma distribution is a flexible distribution that includes the chi-squared, Erlang and exponential distributions as special cases. It starts at the origin and is defined by two parameters, the shape (k) and scale (Θ). The mean is $k\Theta$ while the variance, is $k\Theta^2$; although other ways of defining scale and shape parameters exist (Evans et al., 1993;

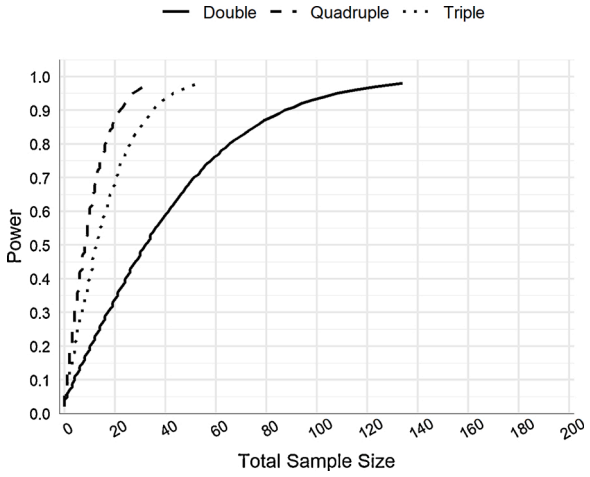

Fig. 2. Power analysis showing how the power changes with the number of animals studied. The total sample size is the total number of animals required, split evenly between two groups. Double, quadruple and triple refers to the number of eosinophils in one group of sheep compared to another.

Papoulis and Pillai, 2002). Fig. 3 illustrates the way the gamma distribution changes as the shape parameter increases for a scale parameter of 60. As the shape parameter increases, the Gamma distribution approaches a normal distribution.

The shape parameter was lower, and the scale parameter was higher, in August compared with September. Consequently, the distribution of eosinophil counts changed from August to September. This could be a consequence of changes in the intensity of nematode infection due to cooling weather or increasing host immunity, but more research would be necessary to confirm this. Previous work by Stear et al. (2002) found that eosinophil counts became closer to a log-normal distribution as the lambs matured. However, they did not determine the drivers of this change (Stear et al., 2002).

The seasonal dynamics of nematode infection and the eosinophil response are reasonably well understood on this farm (Stear et al., 2007) and are assumed to apply to other hill and upland farms in Scotland and Northern England. The dominant nematode species is *Teladorsagia circumcincta* (Stear et al., 1997). Infection is triggered in young lambs in spring by the deposition of nematode eggs during the periparturient rise in mothers but some nematodes probably survive over the winter (Prada Jimenez de Cisneros et al., 2014; Singleton et al., 2011). Infections increase until mid-summer then fall as immune responses develop (Stear et al., 2006) although the precise patterns vary among years (Stear et al., 2006). Eosinophils play an important role in the immune response against nematodes (Stear et al., 1995a) and resistant lambs have more eosinophils (Doligalska et al., 1999; Stear et al., 2002), more IgA (Henderson and Stear, 2006; Strain et al., 2002), more IgE (Murphy et al., 2010) and more discharged mast cells (Stear et al., 1995a). Two major forces drive eosinophilia: response to nematode infection (Stear et al., 1995b) and genetic variation with resistant animals producing more eosinophils (Stear et al., 2002). These forces are antagonistic because resistant animals will have fewer worms to trigger eosinophilia.

The generalized linear modelling has shown that eosinophil counts differed between animals on different fields and between singletons and twins. These differences presumably relate to differences in exposure. Conversely, there were no significant differences between genders or among years. Females do have lower egg counts than castrated males (Abuargob and Stear, 2014) and may be more resistant than males because they produce more nematode specific IgA (Strain et al., 2002). However, females probably acquire fewer nematodes as they are lighter than males and consequently ingest less grass. Possibly decreased exposure and increased resistance between genders counteract each other and lead to similar levels of eosinophilia. There were large differences among years in the mean number of worms recovered at necropsy (Stear et al., 2006) but egg production was similar because of density-dependent effects on fecundity (Stear and Bishop, 1999). The observation that there were no significant differences in eosinophilia, despite large differences in worm number among years, suggests that worm number is not the primary driver of the eosinophil response to infection. Perhaps the eosinophil response is to worm products; because worms are smaller and less fecund in years of high infection, they will produce fewer products per worm.

Power analysis determined the number of animals required to find a significant difference in eosinophil counts between two groups of animals. In general, fewer animals will be needed to detect large effects (Lipsey, 1990). However, quite large sample sizes were required to reliably detect quite large differences in eosinophil counts. A power analysis that assumes a normal distribution for data that is closer to a gamma distribution will not provide accurate indications of the true power (Cundill and Alexander, 2015). Underpowered experiments are likely to suffer from false negative conclusions while overpowered experiments are unnecessarily expensive, can subject animals to unnecessary procedures and are consequently unethical.

In summary, this study determined that variation among animals in eosinophil counts follows a Gamma distribution more closely than a lognormal distribution. Generalized linear modelling with a gamma distribution is more likely to provide reliable inferences than a logarithmic transformation. Generalized linear mixed modelling identified some of the forces that shape the variation among animals and showed variation among animals from different sires and dams as well as differences among animals kept on separate fields and between singletons and twins. A power analysis showed that quite large numbers of animals will be required to detect even quite large differences among groups of animals.

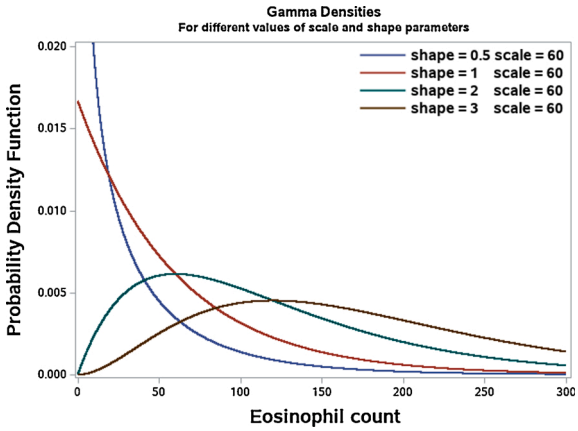

Fig. 3. Density estimates for the Gamma distribution, with a shape parameter ranging from 0.5 to 3 and a scale parameter of 60.

CRediT authorship contribution statement

Caitlin J. Jenvey: Conceptualization, Investigation, Formal analysis, Software, Writing - original draft. **Fazel Almasi:** Software, Writing - review & editing. **Emma U. Halliwell:** Writing - review & editing. **Xia Li:** Formal analysis, Software. **David Piedrafita:** Conceptualization, Writing - review & editing. **Sarah Preston:** Conceptualization, Writing - review & editing. **Michael J. Stear:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abuargob, O., Stear, M.J., 2014. Differences between female and castrated male lambs in susceptibility to natural, predominantly *Teladorsagia circumcincta* infection. Vet. Parasitol.

Cundill, B., Alexander, N.D.E., 2015. Sample size calculations for skewed distributions. BMC Med. Res. Methodol. 15, 28-28.

Dawkins, H., Windon, R., Eagleson, G., 1989. Eosinophil responsiveness in sheep selected for high and low responsiveness to *Trichostrongylus colubriformis*. Int. J. Parasitol. 19, 199–205.

Doligalska, M., Moskwa, B., Stear, M.J., 1999. Relationships among peripheral eosinophilia, eosinophil peroxidase activity, interleukin-5 concentration and faecal nematode egg count during natural, mixed gastrointestinal nematode infection. Vet. Immunol. Immunopathol. 70, 299–308.

Evans, M., Hastings, N., Peacock, B., 1993. Statistical Distributions. Wiley, New York.

Fisher, R.A., 1930. The Genetical Theory of Natural Selection. Oxford University Press, Oxford.

Henderson, N.G., Stear, M.J., 2006. Eosinophil and IgA responses in sheep infected with *Teladorsagia circumcincta*. Vet. Immunol. Immunopathol. 112, 62–66.
Hohenhaus, M.A., Josey, M., Dobson, C., Outteridge, P., 1998. The eosinophil leucocyte, a phenotypic marker of resistance to nematode parasites, is associated with calm behaviour in sheep. Immunol. Cell Biol. 76, 153–158.
Lipsey, M.W., 1990. Design Sensitivity. Statistical Power for Experimental Research. Sage Publications, Newbury Park.
McCullagh, P., Nelder, J.A., 1989. Generalized Linear Models, 2nd edition. Chapman & Hall, London.
Murphy, L., Eckersall, P.D., Bishop, S.C., Pettit, J.J., Huntley, J., Burchmore, R., Stear, M. J., 2010. Genetic variation among lambs in peripheral IgE activity against the larval stages of *Teladorsagia circumcincta*. Parasitology 137, 1249–1260.
Nelder, J.A., Wedderburn, R.W.M., 1972. Generalized linear models. J. Roy. Stat. Soc. Ser. A (Gen) 135, 370–384.
Papoulis, A., Pillai, S., 2002. Probability, Random Variables, and Stochastic Processes, 4th edition. McGraw Hill, New York.
Prada Jimenez de Cisneros, J., Stear, M.J., Mair, C., Singleton, D., Stefan, T., Stear, A., Marion, G., Matthews, L., 2014. An explicit immunogenetic model of gastrointestinal nematode infection in sheep. J. Roy. Soc. Interface Roy. Soc. 11.
R Core Team, 2020. R: a Language and Environment for Statistical Computing. URL. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Rainbird, M., Macmillan, D., Meeusen, E., 1998. Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. Parasite Immunol. 20, 93–103.
Singleton, D.R., Stear, M.J., Matthews, L., 2011. A mechanistic model of developing immunity to *Teladorsagia circumcincta* infection in lambs. Parasitology 138, 322–332.
Stear, M.J., Bishop, S.C., 1999. The curvilinear relationship between worm length and fecundity of *Teladorsagia circumcincta*. Int. J. Parasitol. 29, 777–780.
Stear, M.J., Bishop, S.C., Doligalska, M., Duncan, J.L., Holmes, P.H., Irvine, J., McCririe, L., McKellar, Q.A., Sinski, E., Murray, M., 1995a. Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. Parasite Immunol. 17, 643–652.
Stear, M.J., Bishop, S.C., Duncan, J.L., McKellar, Q.A., Murray, M., 1995b. The repeatability of faecal egg counts, peripheral eosinophil counts, and plasma pepsinogen concentrations during deliberate infections with *Ostertagia circumcincta*. Int. J. Parasitol. 25, 375–380.
Stear, M.J., Bairden, K., Duncan, J.L., Holmes, P.H., McKellar, Q.A., Park, M., Strain, S.A. J., Murray, M., Bishop, S.C., Gettinby, G., 1997. How hosts control worms. Nature 389, 27-27.
Stear, M.J., Henderson, N.G., Kerr, A., McKellar, Q.A., Mitchell, S., Seeley, C., Bishop, S. C., 2002. Eosinophilia as a marker of resistance to *Teladorsagia circumcincta* in Scottish Blackface lambs. Parasitology 124, 553–560.
Stear, M.J., Abuagob, O., Benothman, M., Bishop, S.C., Innocent, G., Kerr, A., Mitchell, S., 2006. Variation among faecal egg counts following natural nematode infection in Scottish Blackface lambs. Parasitology 132, 275–280.
Stear, M.J., Fitton, L.A., Innocent, G.T., Murphy, L., Rennie, K., Matthews, L., 2007. The dynamic influence of genetic variation on the susceptibility of sheep to gastrointestinal nematode infection. J. Roy. Soc. Interface Roy. Soc. 4, 767–776.
Stevenson, L.M., Huntley, J.F., Smith, W.D., Jones, D.G., 1994. Local eosinophil- and mast cell-related responses in abomasal nematode infections of lambs. FEMS Immunol. Med. Microbiol. 8, 167–174.
Strain, S.A.J., Bishop, S.C., Henderson, N.G., Kerr, A., McKellar, Q.A., Mitchell, S., Stear, M.J., 2002. The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep. Parasitology 124.
Terefe, G., Griesz, C., Prevot, F., Bergeaud, J.-P., Dorchies, P., Brunel, J.-C., Francois, D., Fourquaux, I., Jacquiet, P., 2007. In vitro pre-exposure of *Haemonchus contortus* L3 to blood eosinophils reduces their establishment potential in sheep. Vet. Res. 38, 647–654.
Terefe, G., Lacroux, C., Prévot, F., Grisez, C., Bergeaud, J.P., Bleuart, C., Dorchies, P., Foucras, G., Jacquiet, P., 2009. Eosinophils in *Haemonchus contortus*-infected resistant and susceptible breeds of sheep: abomasal tissue recruitment and *in vitro* functional state. Vet. Parasitol. 165, 161–164.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Woolaston, R.R., Manueli, P., Eady, S.J., Barger, I.A., Le Jambre, L.F., Banks, D.J.D., Windon, R.G., 1996. The value of circulating eosinophil count as a selection criterion for resistance of sheep to trichostrongyle parasites. Int. J. Parasitol. 26, 123–126.