SMARTER

SMAll RuminanTs breeding for Efficiency and Resilience

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DELIVERABLE D2.1

Report on new immunological and physiological profiles linked to disease phenotypes in locally-adapted breeds

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About the SMARTER research project

SMARTER will develop and deploy innovative strategies to improve Resilience and Efficiency (R&E) related traits in sheep and goats. SMARTER will find these strategies by: i) generating and validating novel R&E related traits at a phenotypic and genetic level ii) improving and developing new genome-based solutions and tools relevant for the data structure and size of small ruminant populations, iii) establishing new breeding and selection strategies for various breeds and environments that consider R&E traits.

SMARTER with help from stakeholders chose several key R&E traits including feed efficiency, health (resistance to disease, survival) and welfare. Experimental populations will be used to identify and dissect new predictors of these R&E traits and the trade-off between animal ability to overcome external challenges. SMARTER will estimate the underlying genetic and genomic variability governing these R&E related traits. This variability will be related to performance in different environments including genotype-by-environment interactions (conventional, agro-ecological and organic systems) in commercial populations. The outcome will be accurate genomic predictions for R&E traits in different environments across different breeds and populations. SMARTER will also create a new cooperative European and international initiative that will use genomic selection across countries. This initiative will make selection for R&E traits faster and more efficient. SMARTER will also characterize the phenotype and genome of traditional and underutilized breeds. Finally, SMARTER will propose new breeding strategies that utilise R&E traits and trade-offs and balance economic, social and environmental challenges.

The overall impact of the multi-actor SMARTER project will be ready-to-use effective and efficient tools to make small ruminant production resilient through improved profitability and efficiency.

Content

Preamble

Here we give a report on new immunological and physiological profiles linked to disease phenotypes in local breeds of Scottish Blackface sheep, (UK) and 3 locally-adapted goat breeds in Greece. New immunological and physiological profiles in the SBF sheep population were blood lymphocyte cytokine production and serum levels of nematode parasite-specific IgA. In the Greek goat breeds these included somatic cell score and total variable count. The potential of using new udder health phenotypes as predictors of subclinical mastitis in the three semi-extensively reared dairy goat breeds in Greece is described below. Also in Switzerland and Uruguay, a proxy trait for anaemia ('Famacha') has been used on 1250 Lacaune dairy ewes and >1,000 Corriedale ewes respectively, the former of which were also phenotyped for faecal egg counts, packed cell volume (PCV, or Heamatocrit) and milk production traits,

representing the same traits measured in different breeds and in different countries. These latter 2 studies are documented in the peer-reviewed papers reported for SMARTER associated with WP2 and currently, Famacha is being used for management decisions but not yet included into the national breeding programme for sheep in Uruguay. Collectively, these 4 studies use different measures including antibody response, faecal egg counts, anaemia and haematocrit to evaluate novel indicators of disease resistance and resilience in sheep and goats.

Famacha was developed specifically as a proxy trait for Barber's Pole worm (*Haemonchus contortus*). *Haemonchus* is a major problem worm species affecting sheep productivity in Uruguay and is an emerging problem in other parts of the world. The table below shows the total Uruguayan data we have for FAMACHA (in their national database, shown in the 1st first column). The second column shows data that were recorded during SMARTER, which were progeny born between 2018-2021 with their records taken in 2019-2022. (In the third column are the number of animals that have FAMACHA data and feed intake and methane emission simultaneously). Famacha as a proxy trait for anaemia (caused by Haemonchus) was analysed to determine the heritable basis, and would be a meaningful (and cheap and easy) trait to screen large numbers of animals and select animals that are more resistant to the effects of Haemonchus. Currently, Famacha is being used for management decisions but not yet included into the national breeding programme in Uruguay.

Breed	Total	2018-2021	With Feed intake & CH4
Corriedale	7989	4912	309
Australian Merino	2577	987	971
Dohne Merino	1901	1518	354

The main body of this document is the Deliverable D2.1, 'Report on new immunological and physiological profiles linked to disease phenotypes in locally-adapted breeds', which includes the UK and Greek studies in some detail.

Summary

The goals of the SRUC study in Scottish Blackface sheep were to estimate the genetic parameters for blood lymphocyte cytokine production and serum levels of nematode parasite-specific IgA, and assess the relationship between these immune measurements with disease and productivity traits in sheep. Whole blood stimulation assays were used to characterise the adaptive immune response of 1040 lambs measured at 53 days of age in 2016-2017. Blood was stimulated with either pokeweed mitogen (PWM), a lectin that non-specifically activates lymphocytes irrespectively of their antigen specificity, and *Teladorsagia circumcincta* (T-ci) larval antigen to activate parasite-specific T lymphocytes. The type of adaptive immune response was determined by quantifying the cytokines interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-10, which relate to T-helper type 1 (Th1), Th2 and regulatory T cell (Treg) responses, respectively. T-ci specific Immunoglobulin A (IgA) within serum was also quantified. Heritabilities were estimated for each immune trait, and genetic and phenotypic correlations with disease and productivity phenotypes were derived. Disease phenotypes were expressed as faecal egg counts of nematode parasites (Strongyles - FEC_S and Nematodirus - FEC_N) and faecal oocyst counts (coccidian parasites – FOC), and also with a faecal soiling (DAG) score; production was measured as live weight of lambs. Significant genetic variation was observed in both generalised and immune response traits. Heritabilities of cytokine production varied from low (0.14±0.06) to very high (0.77±0.09) and were always significantly greater than zero ($P<0.05$). IgA heritability was found to be moderate. A strong positive correlation was found between FOC and PWM-induced IFN-γ production (IFN-γ_{PWM}) while a stronger but negative correlation was found between FOC and T-ci induced IL-10 (IL-$10_{T\text{-}CI}$). Live weight was negatively genetically correlated with IFN-γ responses. Overall, IFN-γ and IL-4 responses were positively correlated, providing little evidence of cross-regulation of Th1 and Th2 immunity within individual sheep. Furthermore, Immunoglobulin A was highly positively correlated with IL-10_{PWM} and moderately positively correlated with IL-$4_{T\text{-}ci}$. Our results suggest that while selection for high IFN-γ responsiveness may be beneficial for coccidian parasite control,

selection for this trait may negatively affect productivity, which will need to be considered in genetic improvement programmes.

For the Greek study of new udder health phenotypes, the objective was to assess the potential use of new udder health phenotypes as predictors of subclinical mastitis in three semi-extensively reared dairy goat breeds in Greece. A total of 531 dairy goats of two indigenous Greek breeds (Eghoria, n=189 and Skopelos, n=151) and one foreign breed (Damascus, n=191) were used (Figure 1). Animals were randomly selected from seven farms; two with Eghoria, two with Skopelos and three with Damascus goats located in Northern and Central Greece. Udder health traits were recorded monthly for two consecutive milking periods. Specifically, following hand-milking of each goat, a milk sample was collected from both udder halves to be tested for total viable count (TVC). Then, a second milk sample was collected from the milking bucket to assess milk somatic cell count (SCC). Each individual goat that produced milk with SCC >106/mL and TVC >20 × 103/mL during the preceding sampling was selected for the collection of a third milk sample for microbiological analyses. An animal was considered to have subclinical mastitis each time a pathogen was isolated from its milk during bacteriological testing. A new, sub-clinical phenotypic index was devised to categorise animals according to a combination of levels of TVC & SCC to create a new phenotype. Estimates on the association of the studied new udder health phenotypes with subclinical mastitis are presented. Results suggest that udder health phenotypes based on thresholds for milk SCC and TVC at >106 cells/ml and >20×103 cfu/ml, respectively could be used to predict subclinical mastitis in Eghoria, Skopelos and Damascus dairy goats.

Introduction

Gastrointestinal (GI) infections with coccidian (protozoan) and nematode parasites pose a serious constraint on sheep production. Infections with these parasites are widespread in grazing sheep and co-infection with coccidian and nematode parasites is common. While both types of parasite infect the GI tract, there are considerable differences in their interaction with the host, with single cell coccidian parasites

infecting and replicating in epithelial cells and larger multicellular nematode parasites residing within the GI lumen or closely associated with the GI mucosa. The success or failure of immune response of the host to infection depends on a range of different factors, such as pathogen burden and the scale of the immune response itself, with the latter being regulated by the activity of T helper cells. Two of the most important subsets of T helper cells are T helper 1 (Th1) and T helper 2 (Th2), from which naïve T cells differentiate following antigen presentation. Different functions are associated with Th1 and Th2: while the former are primarily involved in inflammatory responses and controlling intracellular pathogens, the latter are mainly involved in inducing humoral responses, typically to extracellular pathogens. Among the cytokines involved in Th1 immune responses are interferon-gamma (IFN-γ), interleukin (IL)-2, and Tumour Necrosis Factor-alpha (TNF-α), whereas Th2 immunity is associated with production of IL-4, IL-5, IL-9 and IL-13 .In the context of ovine GI parasite infections, coccidian parasites are thought to be controlled by Th1 immune responses whereas Th2 cells play a key role in controlling parasitic nematode infections. Of critical importance to the adaptive immune response is another subset of T cells, referred to as regulatory T cells (Treg). Treg are responsible for regulating the immune responses by preventing or inhibiting immune responses, partially through production of inhibitory cytokines such as IL-10 and Transforming Growth Factor-beta (TGF-β). Treg play a key role in preventing over-activation of the immune response and subsequent immunopathology, but may also be actively induced by certain parasitic ovine nematodes as part of their immune evasion strategy. These three Th subtypes (Th1, Th2 and Treg) are characterised by the secretion of key prototypic cytokines following T cell activation. One of the main cytokines produced by Th1 cells is IFN-γ , which is recognised as a key limiting factor in coccidian infections as it promotes Th1 differentiation and is an inhibitor of Th2 cell proliferation. A key cytokine involved in Th2 immune responses against nematodes is IL-4 (McNeilly and Nisbet, 2014). This cytokine is produced by Th2 polarised cells and is involved in promoting antibody responses and B cell class switching to IgE.

Also, Interleukin-10 (IL-10)has been shown to have an immunomodulatory role, controlling and mediating inflammatory responses during infections by a wide range of pathogens including protozoa and nematodes but is also key in controlling autoimmune diseases and allergy amongst other actions.

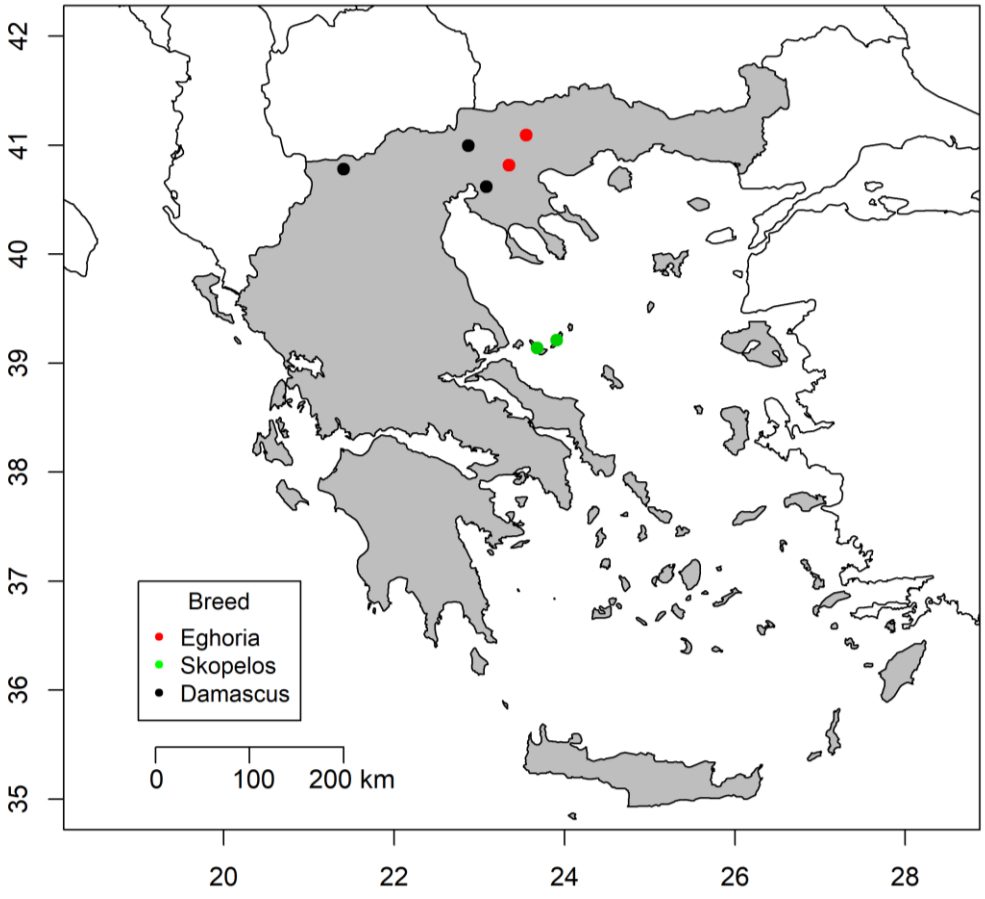
Parasite specific antibodies, in particular IgA, are also key on the immune response against gastrointestinal (GI) parasites. IgA is the most abundant antibody isotype at mucosal surfaces and nematode-specific IgA is known to be linked to resistance to nematodes in sheep, being associated with reduced worm size and fecundity in natural infections.

It has been shown that independent of age, sex, and each other, production of IL-4 from T cell mitogen stimulated whole blood negatively predicted GI nematode faecal egg count in a wild population of Soay sheep, while production of IFN-γ negatively predicted coccidian faecal oocyst. This suggests that Th1 and Th2 immune traits derived from circulating lymphocytes may be useful selection marker for parasite resistance. However, while parasite specific IgA is known to be moderately heritable in sheep, the genetics underlying variation in different types of Th immune responses and how these relate to productivity and disease is currently unknown.

In Greece**,** 7 farms and with 531 dairy goats of two indigenous Greek breeds (Eghoria, n=189 and Skopelos, n=151) and one foreign breed (Damascus, n=191) were used for the study (Figure 1). Animals were randomly selected from seven farms; two with Eghoria, two with Skopelos and three with Damascus goats located in Northern and Central Greece (Figure 2). Goat herds were representative of the typical farming systems in these areas. These are low-input pastoral farming systems characterised by grazing throughout the year, random mating and minor differences in management practices between herds.

Figure 1. Eghoria (a), Skopelos (b) and Damascus (c) dairy goat breeds used in the study.

Figure 2. Map of Greece illustrating the regions in which the studied goat herds were located.



Aims of study

The aims of the UK study were to (i) evaluate T cell cytokine production from whole blood (as a measure of Th polarisation) and nematode parasite specific IgA levels in lambs, (ii) examine the host genetic background for these traits and (iii) assess their relationship with animal disease and production traits. For this study, the immune status of more than 1,000 pedigree sheep was determined, and we estimated the amount of genetic variance between animals and the trait heritability, and derived genetic and phenotypic correlations with parasitic infection phenotypes and live weight of animals.

The overall objective of the Greek mastitis study was to assess the potential use of new udder health phenotypes as predictors of subclinical mastitis in three semi-extensively reared dairy goat breeds in Greece. Specifically, the aims of the study are to quantify the relationships between udder health phenotypes and subclinical mastitis in each of the three breeds described above.

Methodology

Scottish Blackface sheep study

Animals and traits

Blood samples for cellular and serum antibody analyses were collected from a total of 1040 Scottish Blackface lambs at two months of age, born in 2016 and 2017, belonging the SRUC experimental hill farm flock, Midlothian, Scotland. Faecal samples were collected from the same individuals one month later. Animals were managed in typical hill farm conditions throughout the year and continually exposed to natural GI infections. The flock has been continually monitored since 1990 for aspects of performance and health from which several genetic studies have been published.

At the first sampling time-point, whole blood stimulation assays were used to characterise the adaptive immune response traits of this flock in response to Pokeweed mitogen (PWM) and the common GI nematode *Teladorsagia circumcincta*

(T-ci) by measuring release of the cytokines interferon-gamma (IFN-γ), interleukin (IL)-4, and IL-10, which relate to T-helper type 1 (Th1), Th2 and regulatory T cell (Treg), respectively. PWM is a mitogenic lectin which stimulates B and T lymphocytes irrespective of antigenic specificity , while *T. circumcincta* somatic antigen from fourth stage larvae (Tci-L4) was used to activate parasite specific lymphocytes. Levels of *T. circumcincta* specific immunoglobulin (Ig)A in serum were also quantified by ELISA. Additionally, at the second sampling time-point individual animal data were collected on faecal counts of Strongyle (FEC_S) and Nematodirus (FEC_N) eggs and Coccidia oocysts (FOC), along with records on faecal soiling (DAG score) and live weight (LWT) as described in Pacheco et al, (2021). Designations for the different cytokines and respective stimulants are shown in Table 1.

Whole blood stimulation assays

Blood was collected aseptically into serum and lithium heparin vacutainers (Becton Dickinson, Oxford, UK) by jugular venepuncture. Whole blood stimulation assays were carried out by mixing 100µl of whole blood with 100µl of complete medium [RPMI-1640 (Gibco, ThermoFisher Scientific) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin and 50 µM 2-mercaptoethanol (all from Sigma-Aldrich, UK)] containing 10µg/ml final concentration of PWM, 5µg/ml T-ci-l4 or phosphate buffered saline (PBS) as control to account for any non-specific cytokine secretion. Samples were plated in triplicate in tissue culture grade round bottom 96-well plates (Corning Costar, Sigma-Aldrich, UK). The plates were then incubated at 37°C with 5% of CO_2 in air for 48h. After the incubation period, plates were spun at 1500rpm for 5min and supernatants stored at -20°C for cytokine analysis.

Cytokine ELISA

Capture ELISAs were performed to examine the secretion of IFN-γ, IL-4 and IL-10, following stimulation with PWM or T-ci-L4. All incubations were carried out at room temperature unless stated otherwise. IL-4 and IFN-γ were quantified using commercial ELISA kits according to the manufacturer's instructions (MABTECH AB, Augustendalsvägen, Sweden). Mouse monoclonal anti-bovine IL-10 capture and detection antibodies (clones CC318 and CC320b, respectively, BioRad, UK) and standard curves produced using supernatants from COS-7 cells transfected with

bovine IL-10 were used to quantify IL-10 secretion. Washing steps for all ELISAs were performed 6 times with 350µl washing buffer (Phosphate Buffered Saline (PBS) + 0.05% Tween20) using a Thermo Scientific Wellwash™ Versa (ThermoFisher Scientific). High-binding capacity ELISA plates (Immunolon™ 2HB 96-well microtiter plates, ThermoFisher Scientific) were incubated with coating antibodies overnight at 4°C. Plates were then washed and blocked for 1h with PBS containing 0.05% Tween 20 (Sigma-Aldrich, UK) and 0.1% BSA Bovine Serum Albumin (BSA, Sigma-Aldrich, UK) for IL-4, IFN-γ or PBS containing 3% of BSA for IL-10. Following a further washing step, 50µl of supernatants or standards were added in duplicate for 1h. Subsequently, plates were washed and detection antibodies added for 1h. This was followed by washing and addition of Streptavidin-HRP (Dako, Agilent, Santa Clara, US) for 45 min. After the final washing step, SureBlue TMB substrate (Insight Biotechnology, London, UK) was added and the reaction was stopped by the addition of TMB stop solution (Insight Biotechnology, London, UK). Absorbance values were read at O.D. 450nm. Standard curves were included in all plates and were constructed using seven serial dilutions of recombinant cytokines ranging from 400 to 6.25 pg/ml for IFN- γ (MABTECH AB); 2,000 to 62.5pg/ml for IL-4 (MABTECH AB) and 13.2 to 0.206 BU/ml for IL-10.

Ovine T. circumcincta specific IgA ELISA

Indirect ELISAs were carried out to detect antigen specific T-ci IgA in serum samples. Briefly, high-binding capacity ELISA plates (Immunolon™ 2HB 96-well microtiter plates, ThermoFisher Scientific) were incubated with 5µg/ml of parasite antigen (*T. circumcincta* L3 somatic antigen in 0.5M bicarbonate buffer, pH 9.6) at 4°C overnight. Washing steps were carried out as detailed for cytokine ELISAs and incubations carried out at 37°C unless stated differently. Following overnight incubation, plates were washed and blocked with 200 µl of blocking buffer (PBS plus 3% fish gelatin, Sigma-Aldrich, UK) for 1h. Following a further washing step, sera samples diluted 1:4 in dilution buffer (PBS+ 0.5% Tween80+ 0.5M NaCl) were added in duplicate and incubated for 1h. Each plate also included a positive control serum sample. Following washing, 100µl of 1:15000 polyclonal rabbit anti-ovine IgA conjugated to horse radish peroxidase (AHP949P, BioRad, UK) was added to all wells and incubated for 1h. After a final wash, 100 µl of TMB substrate (TMB substrate kit, ThermoFisher Scientific) was

added and reaction stopped after 5 min by the addition of TMB stop solution provided within the TMB substrate kit. Absorbance values were read at O.D. 450nm. All values were then normalised using the positive control.

Data analysis

Preliminary analyses were carried out to determine significant fixed effects affecting the immunological traits of study. Subsequently, the following mixed models were used for the statistical analysis:

$$y = X\beta + Za + e,$$

where:

- y is the trait record (IFN-γ, IL-4, IL-10 and IgA).
- β is the vector of significant fixed effects
- a is the vector of additive genetic effects, including the animal pedigree
- e is the vector of random residual effects
- X and Z are the design matrices linking records to fixed or random effects.

The fixed effects used in this study included sex of the animal, grazing location birth-rearing rank (single or twins), year of birth, genetic line, and age of dam at parturition. When appropriate, significant interactions were also fitted. Immunological data was log-transformed (Log +1) prior to all analyses in order to ensure normality of distribution.

Each immunological trait was first analysed separately to derive estimates of genetic variance and trait heritability. Subsequently, a series of bivariate analyses were conducted to estimate genetic and phenotypic correlations among the immune traits, and between immune traits and disease (FEC_S, FEC_N, FOC, DAG) and production (LWT) traits.

All analyses were performed using ASReml v3.0 (Gilmour et al., 2009) statistical software.

Greek goat study

The methodology for the Greek study used historical data, which were collected during FP7-SOLID project (Sustainable Organic Low Input Dairying, 266367, 2012-2014). Udder health traits were recorded monthly for two consecutive milking periods. Specifically, following hand-milking of each goat, a milk sample was collected from both udder halves to be tested for total viable count (TVC). Then, a second milk sample was collected from the milking bucket to assess milk somatic cell count (SCC). Each individual goat that produced milk with SCC $>10^6$/mL and TVC $>20 \times 10^3$/mL during the preceding sampling was selected for the collection of a third milk sample for microbiological analyses. An animal was considered to have subclinical mastitis each time a pathogen was isolated from its milk during bacteriological testing. Sampling and analyses of milk samples have been described in detail in previous studies (Gelasakis et al., 2016, 2018). Briefly, microbiological analyses involved isolation of coagulase-negative staphylococci, coagulase-positive staphylococci, streptococci/enterococci, other Gram-positive bacteria, Gram-negative bacteria and *Mycoplasma agalactiae*.

Based on SCC and TVC records a subclinical mastitis index (SMI) was defined using the following formula:

$$SMI = 0.6\frac{SCC - Mean}{SD} + 0.4\frac{TVC - Mean}{SD}$$

Where:

Mean and SD correspond to the mean and standard deviation, respectively of each trait (SCC and TVC) within each studied breed.

Moreover, thresholds set for SCC and TVC at $>10^6$ cells/ml and $>20\times10^3$ cfu/ml, respectively were used to define three additional new udder health phenotypes: i) UHP1, scored as 0 or 1 if at least one of the traits was below or both were above thresholds, respectively, ii) UHP2, scored as 0-2 if both traits were below thresholds, one of the two was above, or both exceeded thresholds, iii) UHP3 scored as 0-3 if both traits were below, only TVC was above, only SCC was above, or both were above thresholds.

Descriptive statistics of the data used in the study are shown in Tables 1 and 2.

Table 1. Frequency (%) of subclinical mastitis and udder health phenotypes UHP1, UHP2 and UHP3 (corresponding number of observations in parenthesis) in the studied goat breeds.

		Breed			
Trait	**Levels**	**Eghoria**	**Skopelos**	**Damascus**	**Total**
Subclinical mastitis (0-1)	0	32.40 (127)	12.39 (41)	43.63 (185)	30.78 (353)
	1	67.60 (265)	87.61 (290)	56.37 (239)	69.22 (794)
UHP1 (0-1)	0	33.89 (122)	32.30 (94)	25.99 (72)	31.03 (288)
	1	66.11 (238)	67.70 (197)	74.01 (205)	68.97 (640)
UHP2 (0-2)	0	10.83 (39)	11.34 (33)	7.58 (21)	10.02 (93)
	1	23.06 (83)	20.96 (61)	18.41 (51)	21.01 (195)
	2	66.11 (238)	67.60 (197)	74.01 (205)	68.97 (640)
UHP3 (0-3)	0	10.83 (39)	11.34 (33)	7.58 (21)	10.02 (93)
	1	5.28 (19)	1.37 (4)	6.50 (18)	4.42 (41)
	2	17.78 (64)	19.59 (57)	11.91 (33)	16.59 (154)
	3	66.11 (238)	67.70 (197)	74.01 (205)	68.97 (640)

UHP1= scored as 0 if somatic cell count ≤10^6 cells/ml and/or total viable count ≤20×10^3 cfu/ml, or 1 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml; UHP2= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤20×10^3 cfu/ml, 1 if somatic cell count >10^6 cells/ml or total viable count >20×10^3 cfu/ml, respectively, or 2 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml; UHP3= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤20×10^3 cfu/ml, 1 if only total viable count >20×10^3 cfu/ml, 2 if only somatic cell count ≤10^6 cells/ml or 3 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml.

Table 2. Descriptive statistics of subclinical mastitis index in the studied goat breeds.

Breed	**N**	**Mean**	**SD**	**Min**	**max**
Eghoria	360	0.63	1.25	-0.48	5.01
Skopelos	291	0.97	1.50	-0.44	5.56
Damascus	277	0.58	1.17	-0.57	4.39
Total	928	0.72	1.32	-0.57	5.56

Statistical analyses

Preliminary analyses were performed to identify environmental factors with significant effects on subclinical mastitis. The effects of farm, period of kidding, age at kidding, days from kidding and interactions between them were tested. The association of UHP1, UHP2 and UHP3 with subclinical mastitis (binary trait) was tested with mixed non-linear models, which included all significant effects from the preliminary analyses and a logit function for binomial distribution:

$$Y_{ijkmn} = \mu + F_iP_j + b1*D + UHP_k + A_m + e_{ijkmn,} \quad (1)$$

Where:

Y_{ijkmn}= subclinical mastitis (n^{th} measurement on animal m);
μ= overall population mean;

F_iP_j= fixed effect of the interaction between farm (7 levels) and period of kidding (2 levels);
b1= regression coefficient on days from kidding (D);
UHP_k= fixed effect of each udder health phenotype (tested separately; UHP1: 2 levels, UHP2: 3 levels, UHP3: 4 levels);
A_m= random effect of the animal (m=1-531 goats);
e_{ijkmn}= random residual effect.

Then, the regression on SMI was fitted in the above model instead of the udder health phenotype effect to test its association with subclinical mastitis.

The above analyses were performed using the statistical package “lme4” in R programming language (Bates et al. 2015) and the level of statistical significance was set at 0.05.

In a separate series of bivariate analyses, animal correlations of subclinical mastitis with each studied udder health phenotype were estimated with bayesian Markov Chain Monte Carlo methods implemented in the R software package “MCMCglmm” (Hadfield, 2010) and using the AUTH Compute Infrastructure and Resources. The bivariate models included the same environmental factors as model (1) and also the regression coefficient on age at kidding in the case of all udder health phenotypes.

Weekly informative priors were used for random and residual effects, whereas for the fixed effects the default normal prior distribution with null mean and a large variance (1e+10) was used (Hadfield, 2010). For each model, three chains of 1,003,000 to 40,003,000 iterations with a burn-in period of 3,000 iterations and a thinning interval of 1,000 to 40,000 samples were used (depending on each model’s convergence and autocorrelation diagnostics). Convergence of the models was tested with visual inspection of estimate plots and the Gelman and Rubin’s convergence diagnostic where values above 1 indicate lack of convergence (Gelman and Rubin, 1992). Moreover, autocorrelation across chains was tested for all lag values greater than zero (values below 0.1 were considered acceptable). Then, animal correlations between studied traits were estimated from the posterior means of corresponding covariance values after convergence.

Both types of analyses were performed within and across-breed. Finally, since breed was confounded within farms, the farm effect was essentially a farm-breed combination effect in all across-breed analyses.

Results

Scottish Blackface sheep study

For the UK study, heritability estimates of cytokine expression (Table 2) varied considerably between cytokine types and the stimulation assay. The biggest difference pertained to IL-4 (0.77±0.09 vs. 0.14±0.06, for PWM and T-ci, respectively). All estimates were significant ($P<0.05$).

Table 3 summarises estimates genetic correlations (r_G) between traits. We found a strong and positive r_G between FOC and IFN-γ $_{PWM}$ (0.67±0.30), and a stronger, but in this case, negative r_G FOC and IL-10 $_{T\text{-}ci}$. Live weight (LWT) had an overall negative r_G with IFN-γ, with significant negative genetic correlations between LWT and both IFN-γ $_{PWM}$ and IFN-γ $_{T\text{-}ci}$, indicating a negative effect of Th1 responses on productivity. Overall, Th1 and Th2 were positively genetically correlated (0.57±0.15 between IFN-γ $_{PWM}$ and IL-4 $_{PWM}$; 0.74±0.21 between IFN-γ $_{T\text{-}ci}$ and IL-4 $_{T\text{-}ci}$; and 0.50±0.15 between IFN-γ $_{T\text{-}ci}$ and IL-4 $_{PWM}$), and consequently, partly under the same genetic control. We also found some evidence of genetic correlations between Th2 and regulatory immune responses (0.53±0.23 between IL-10 $_{PWM}$ and IL-4 $_{T\text{-}ci}$). Finally, IgA was moderately correlated with IL-4 $_{PWM}$ (0.32±0.17) and strongly correlated with IL-10 $_{PWM}$ (0.85±0.17).

In the case of phenotypic correlations (r_P, Table 4) we found no significant correlations between immune and disease traits, but there was evidence of antagonism between IFN-γ $_{PWM}$ and LWT (-0.09±0.04), and a positive association between IL-10 $_{PWM}$ and LWT (0.10±0.04), although these correlations were weak. There was also evidence that Th1 and Th2 responses were positively correlated at the phenotypic level. IgA was found to be positively correlated with all cytokines released following polyclonal T cell activation with PWM at the phenotypic level.

Table 1 - Cytokine and stimulant combination designations

Stimulant	Cytokine	Designation
Pokeweed mitogen (PWM)	IFN-γ	IFN-γ $_{PWM}$
	IL-4	IL-4 $_{PWM}$
	IL-10	IL-10 $_{PWM}$
T. circumcincta L4 antigen (T-ci-L4)	IFN-γ	IFN-γ $_{T\text{-}ci}$
	IL-4	IL-4 $_{T\text{-}ci}$
	IL-10	IL-10 $_{T\text{-}ci}$

Table 2 - Immune trait heritabilities (h^2).

Traits	h^2
IFN-γ $_{PWM}$	0.33±0.10
IL-4 $_{PWM}$	0.77±0.09
IL-10 $_{PWM}$	0.16±0.07
IFN-γ $_{T\text{-}ci}$	0.27±0.08
IL-4 $_{T\text{-}ci}$	0.14±0.06
IL-10 $_{T\text{-}ci}$	0.22±0.08
IgA	0.41±0.09

Table 3 - Genetic correlations (r_G) between immunological traits, disease traits (FEC_S, FEC_N, FOC and DAG) and production traits (LWT). (S.E.).

	Genetic correlations						
	IFN-γ $_{PWM}$	IL-4 $_{PWM}$	IL-10 $_{PWM}$	IFN-γ $_{T\text{-}ci}$	IL-4 $_{T\text{-}ci}$	IL-10 $_{T\text{-}ci}$	IgA
FEC_S	-0.20 (0.34)	-0.18 (0.24)	0.01 (0.33)	-0.27 (0.33)	0.01 (0.40)	-0.16 (0.34)	-0.17 (0.32)
FEC_N	-0.16 (0.43)	0.17 (0.33)	-0.16 (0.44)	0.02 (0.40)	0.01 (0.51)	0.20 (0.42)	0.29 (0.40)
FOC	0.67 (0.30)*	-0.17 (0.27)	0.51 (0.41)	-0.28 (0.35)	-0.09 (0.48)	-0.84 (0.31)*	0.59 (0.39)
DAG	0.10 (0.38)	0.39 (0.24)	-0.43 (0.34)	0.34 (0.33)	0.31 (0.46)	-0.03 (0.40)	0.27 (0.31)
LWT	-0.54 (0.18)*	-0.11 (0.18)	0.03 (0.26)	-0.51 (0.20)*	-0.26 (0.32)	0.02 (0.27)	-0.07 (0.25)
IFN-γ $_{PWM}$	–	–	–	–	–	–	–
IL-4 $_{PWM}$	0.57 (0.15)*	–	–	–	–	–	–
IL-10 $_{PWM}$	0.36 (0.28)	0.23 (0.29)	–	–	–	–	–
IFN-γ $_{T\text{-}ci}$	0.19 (0.25)	0.50 (0.15)*	-0.22 (0.24)	–	–	–	–
IL-4 $_{T\text{-}ci}$	-0.06 (0.33)	0.41 (0.26)	-0.53 (0.23)*	0.74 (0.21)*	–	–	–
IL-10 $_{T\text{-}ci}$	0.00 (0.27)	0.03 (0.20)	-0.37 (0.26)	0.25 (0.25)	0.01 (0.35)	–	–
IgA	0.39 (0.23)	0.32 (0.17)*	0.85 (0.17)*	-0.06 (0.25)	-0.15 (0.32)	0.43 (0.23)	–

**Results statistically significant (P<0.05).*

*Table 4 - Phenotypic correlations (r_P) between immunological traits, disease traits (FEC_S, FEC_N, FOC and DAG) and production traits (LWT). (S.E.). *Results statistically significant (P<0.05).*

	Phenotypic correlations						
	IFN-γ PWM	IL-4 PWM	IL-10 PWM	IFN-γ T-ci	IL-4 T-ci	IL-10 T-ci	IgA
FEC_S	-0.04 (0.04)	-0.04 (0.04)	-0.03 (0.04)	-0.05 (0.03)	-0.04 (0.03)	-0.03 (0.04)	0.00 (0.04)
FEC_N	0.04 (0.04)	-0.06 (0.04)	-0.05 (0.03)	-0.06 (0.03)	-0.04 (0.03)	-0.03 (0.03)	-0.03 (0.04)
FOC	-0.01 (0.04)	-0.05 (0.04)	0.04 (0.04)	0.03 (0.04)	-0.02 (0.03)	-0.01 (0.04)	0.03 (0.04)
DAG	0.02 (0.04)	0.06 (0.04)	0.01 (0.03)	0.04 (0.03)	0.03 (0.03)	-0.02 (0.03)	0.05 (0.04)
LWT	-0.09 (0.04)*	0.07 (0.04)	0.10 (0.04)*	0.01 (0.04)	-0.02 (0.36)	0.05 (0.04)	-0.04 (0.04)
IFN-γ PWM	–	–	–	–	–	–	–
IL-4 PWM	0.32 (0.04)*	–	–	–	–	–	–
IL-10 PWM	-0.03 (0.04)	0.13 (0.04)*	–	–	–	–	–
IFN-γ T-ci	0.24 (0.04)*	0.31 (0.04)*	-0.03 (0.04)	–	–	–	–
IL-4 T-ci	0.08 (0.04)*	0.18 (0.04)*	-0.04 (0.04)	0.34 (0.03)*	–	–	–
IL-10 T-ci	-0.01 (0.04)	0.06 (0.04)	0.18 (0.04)*	0.24 (0.03)*	0.06 (0.03)	–	–
IgA	0.08 (0.04)*	0.14 (0.04)*	0.11 (0.04)*	0.02 (0.04)	0.07 (0.04)	0.07 (0.04)	–

Greek goat study

For the Greeek study results, estimates on the association of the studied new udder health phenotypes with subclinical mastitis are presented in Table 3. In Eghoria and Skopelos goats, statistically significant positive associations were reported with UHP2 and UHP3 (P<0.05). Specifically, Eghoria goats scored as 2 for UHP2 and 3 for UHP3 (SCC>106 cells/ml and TVC>20×103 cfu/ml) were in both cases 3.73 times more likely to have subclinical mastitis compared to goats scored as 0 (SCC≤106 cells/ml and TVC≤20×103 cfu/ml). Similarly, Skopelos goats scored as 2 for UHP2 and 3 for UHP3 were more likely to have subclinical mastitis compared to goats scored as 0 (5.30 and 5.36, respectively). No significant associations (P>0.05) were observed when comparisons were made between the other scores of these phenotypes in Eghoria and Skopelos goats, nor with any trait in Damascus goats. Across-breed, statistically significant associations were reported with UHP1, UHP2 and UHP3 (P<0.01). The likelihood of subclinical mastitis was higher by 1.82 times for score 1 in UHP1, 4.26 and 3.46 times for scores 2 and 1, respectively in UHP2 and 4.24 and 3.70 times for scores 3 and 2, respectively in UHP3 compared to score 0.

Table 3. Association of studied udder health phenotypes with presence of subclinical mastitis.

		Eghoria		Skopelos		Damascus		Total	
Trait	**Contrast**	**OR (SE)**	**P**	**OR (SE)**	**P**	**OR (SE)**	**P**	**OR (SE)**	**P**
UHP1 (0-1)	1-0	1.63 (0.53)	0.132	2.20 (1.12)	0.123	1.36 (0.65)	0.521	1.82 (0.45)	0.017
UHP2 (0-2)	2-0	3.73 (1.91)	0.028	5.30 (3.27)	0.019	3.90 (3.59)	0.303	4.26 (1.60)	<0.001
	2-1	1.15 (0.43)	0.922	1.17 (0.70)	0.962	1.06 (0.55)	0.994	1.23 (0.35)	0.741
	1-0	3.23 (1.80)	0.090	4.52 (3.10)	0.072	3.68 (3.52)	0.359	3.46 (1.40)	0.006
UHP3 (0-3)	3-0	3.73 (1.90)	0.048	5.36 (3.31)	0.033	3.75 (3.46)	0.478	4.24 (1.58)	<0.001
	3-1	1.64 (1.10)	0.881	1.97 (3.07)	0.972	1.48 (1.13)	0.955	1.50 (0.75)	0.849
	3-2	1.03 (0.42)	0.999	1.12 (0.69)	0.998	0.84 (0.54)	0.993	1.15 (0.37)	0.973
	2-0	3.60 (2.11)	0.126	4.81 (3.41)	0.120	4.47 (4.51)	0.446	3.70 (1.58)	0.012
	2-1	1.59 (1.17)	0.923	1.77 (2.80)	0.984	1.77 (1.66)	0.929	1.31 (0.73)	0.964
	1-0	2.27 (1.77)	0.719	2.72 (4.23)	0.918	2.53 (2.87)	0.846	2.83 (1.64)	0.276
SMI	NA	1.15 (1.15)	1.15	1.22 (1.22)	0.291	1.19 (1.19)	0.303	1.17 (1.17)	0.0747

OR=odds ratio; SE=standard error; UHP1= scored as 0 if somatic cell count ≤10^6 cells/ml and/or total viable count ≤$20×10^3$ cfu/ml, or 1 if somatic cell count >10^6 cells/ml and total viable count >$20×10^3$ cfu/ml; UHP2= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤$20×10^3$ cfu/ml, 1 if somatic cell count >10^6 cells/ml or total viable count >$20×10^3$ cfu/ml, respectively, or 2 if somatic cell count >10^6 cells/ml and total viable count >$20×10^3$ cfu/ml; UHP3= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤$20×10^3$ cfu/ml, 1 if only total viable count >$20×10^3$ cfu/ml, 2 if only somatic cell count ≤10^6 cells/ml or 3 if somatic cell count >10^6 cells/ml and total viable count >$20×10^3$ cfu/ml; SMI=subclinical mastitis index.

Animal correlations between subclinical mastitis and the studied udder health phenotypes are presented in Table 2. Significant positive correlations (P<0.05) were reported with all traits except for UHP1 in Eghoria and Skopelos goats. Significant estimates ranged from 0.23 to 0.41, 0.24 to 0.38 and 0.44 to 0.66 in Eghoria, Skopelos and Damascus goats, respectively. Across-breed, the highest correlation was reported with UHP2 (0.59), followed by UHP3 (0.51), SMI (0.32) and UHP1 (0.30).

Table 2. Animal correlations (standard error in parenthesis) of subclinical mastitis with the studied udder health phenotypes.

Trait	Eghoria	Skopelos	Damascus	Total
UHP1 (0-1)	0.21 (0.149)	0.25 (0.138)	0.32 (0.140)	0.30 (0.086)
UHP2 (0-2)	0.40 (0.172)	0.38 (0.159)	0.58 (0.265)	0.59 (0.107)
UHP3 (0-3)	0.41 (0.154)	0.35 (0.156)	0.66 (0.161)	0.51 (0.097)
SMI	0.23 (0.106)	0.24 (0.117)	0.44 (0.146)	0.32 (0.077)

UHP1= scored as 0 if somatic cell count ≤10^6 cells/ml and/or total viable count ≤20×10^3 cfu/ml, or 1 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml; UHP2= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤20×10^3 cfu/ml, 1 if somatic cell count >10^6 cells/ml or total viable count >20×10^3 cfu/ml, respectively, or 2 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml; UHP3= scored as 0 if somatic cell count ≤10^6 cells/ml and total viable count ≤20×10^3 cfu/ml, 1 if only total viable count >20×10^3 cfu/ml, 2 if only somatic cell count ≤10^6 cells/ml or 3 if somatic cell count >10^6 cells/ml and total viable count >20×10^3 cfu/ml; SMI=subclinical mastitis index.

Discussion

The results from the UK study show that there is significant genetic variability in all immunological traits investigated in this study, suggesting that individual animals vary in their genetic capacity to mount adaptive immune responses under similar conditions of natural parasite infection. Heritability estimates for *T. circumcincta* specific IgA were similar to those reported previously whereas significant heritability estimates reported for the cellular (cytokine) traits have not been previously reported in sheep. Contrary to our expectations that Th1- and Th2-immunity would negatively regulate each other, we found that Th1 and Th-2 associated cytokine measures were positively correlated. We found no evidence of any association between any of the immune measurements and nematode FEC at either the genetic or phenotypic levels,but did see some associations between IFN-γ and IL-10 release and FOC at the genetic level. Importantly, significant negative genetic correlations were found between IFN-γ production and live weight, suggesting that selection for higher IFN-γ production would come with productivity costs.

Our results show that there is substantial genetic variability among individual lambs with regards to all immunological traits, although it is not clear if selecting for these traits is favourable in regards to live weight. Our results shed light on the complex mechanism of the adaptive immune response in growing lambs. Firstly, we found evidence that both Th1 and Th2 immune responses are partially under the same genetic control, demonstrating the lack of a clear Th1/Th2 dichotomy. Furthermore, consistent with other studies in non-laboratory settings, there was no marked biased polarisation towards a specific immune response. Additionally, there is evidence to suggest that Th1 immune responses at 2 months of age could be impacting the capacity for the animal to gain weight, translating in animals with lower weights at 3 months. Our results form the basis of future studies that continue to build upon the ground work laid here, including exploring the timing of adaptive immune and parasitology trait measurements, and their association with parasite resistance and productivity.

Results obtained in the Greek dairy goat study suggest that udder health phenotypes based on thresholds for milk SCC and TVC at >106 cells/ml and >20×103 cfu/ml, respectively could be used to predict subclinical mastitis in Eghoria, Skopelos and Damascus dairy goats. Amongst the studied phenotypes and according to all performed analyses, UHP2 and UHP3 seem to be the best indicators for evaluating udder health of semi-extensively reared dairy goat breeds in Greece.

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