The impact of SNP density on quantitative genetic analyses of body size traits in a wild population of Soay sheep

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Abstract

Understanding the genetic architecture underpinning quantitative traits in wild populations is pivotal to understanding the processes behind trait evolution. The 'animal model' is a popular method for estimating quantitative genetic parameters such as heritability and genetic correlation and involves fitting an estimate of relatedness between individuals in the study population. Genotypes at genome-wide markers can be used to estimate relatedness; however, relatedness estimates vary with marker density, potentially affecting results. Increasing density of markers is also expected to increase the power to detect quantitative trait loci (QTL). In order to understand how the density of genetic markers affects the results of quantitative genetic analyses, we estimated heritability and performed genome-wide association studies (GWAS) on five body size traits in an unmanaged population of Soay sheep using two different SNP densities: a dataset of 37,037 genotyped SNPs, and an imputed dataset of 417,373 SNPs. Heritability estimates did not differ between the two SNP densities, but the high-density imputed SNP dataset revealed four new SNP-trait associations that were not found with the lower density dataset, as well as confirming all previously-found QTL. We also demonstrated that fitting fixed and random effects in the same step as performing GWAS is a more powerful approach than pre-correcting for covariates in a separate model.

Introduction

Investigating the genetic architecture behind heritable traits is key to understanding the biological diversity of wild populations. If we know the number of loci influencing a trait and their effect size, we can better understand the evolutionary processes that underpin traits, improve inferences about trait evolution, and understand micro-evolutionary dynamics that occur due to environmental change (Barton and Keightley 2002). Most quantitative genetic research in animals is carried out in artificial populations; either domestic, agricultural or laboratory. Such populations experience controlled environmental conditions which make it easier to account for environmental factors when studying the effect of genetic variants on phenotypic variation. However, given that environmental factors can influence the phenotype of a quantitative trait (Charmantier et al. 2014), and that the presence of genotype-by-environment interactions can cause additive genetic variance to differ between environmental conditions, studies on artificial populations arguably cannot be fully extrapolated to wild populations (Kruuk et al. 2008). Therefore, it is important to also study quantitative traits in wild populations in their natural habitats. There is a wealth of quantitative genetics research in human populations (for examples, see Manolio et al. 2009; Kang et al. 2010; Yang et al. 2010; Zaitlen et al. 2013; Locke et al. 2015; Xia et al. 2016; Xia et al. 2021), but humans also arguably experience a more buffered environment than wild populations and inferences in wild populations are lacking in comparison.

When using molecular markers to inform quantitative genetic analyses, the results are dependent on the genetic polymorphisms used in the analysis: low numbers of polymorphisms can result in downwardly biased heritability estimates and regions containing causal variants may not appear as significant if there are no genotyped polymorphisms in linkage disequilibrium (LD) with the causal variant. Increasing the density of genotyped polymorphisms means they are more likely to be in LD with causal variants for the trait of interest, either by being physically closer to the causal variants or by matching the allele frequency of the causal variants more accurately. However, increasing the number of genotyped markers means larger, denser genotyping arrays with costs increasing with density. For commonly studied species, high-density arrays are more affordable due to high demand, but for more niche species, including wild populations, large genotyping arrays are often unaffordable. Genotyping-by-sequencing, e.g. ddRAD (Peterson et al. 2012) is a potentially useful alternative for upscaling SNP density, though the combination of bioinformatics and samples sizes required in quantitative genetic research means that this approach is not yet in widespread use.

As an alternative to expensive high-density genotyping, genotype imputation can be used to increase the number of variants analysed (Burdick et al. 2006). Imputation involves predicting genotypes at untyped SNPs in a 'target' population using a subset of the study population – or more generally a "reference" population – genotyped at a higher density, either through a high density SNP array or by genotyping-by sequencing. The genotypes at these untyped SNPs for individuals in the target population are inferred using their genotypes at typed markers and taking advantage of existing linkage disequilibrium (LD) between SNPs. Pedigree information can also be used to increase the accuracy of the imputation by identifying haplotype blocks that are identical by descent (Burdick et al. 2006).

The Soay sheep *(Ovis aries)* of St Kilda are a primitive, unmanaged breed of sheep that have been the focus of a longitudinal, individual-based study since 1985 (Clutton-Brock and Pemberton 2003). As part of the study, morphometric, life history and environmental data are collected, DNA samples are collected, and a pedigree has been constructed using observation and genetic parentage inference. 7630 sheep have been genotyped on the Ovine SNP50 Illumina Beadchip, on which 37,037 SNPs are autosomal and polymorphic in this population. In addition, 188 individuals have been genotyped on the Ovine Infinum HD Beadchip, which contains 419,281 autosomal SNPs that are polymorphic in the population – this has allowed for imputation of the remaining sheep to this higher density (Stoffel et al. 2021).

In this study we performed a direct comparison of heritability estimates and GWAS associations between the lower density SNP data and the imputed high density SNP data in the Soay population, focusing on five body size traits in neonates, lambs and adults. We performed GWAS by fitting fixed and random effects in the same step as testing for SNP-trait associations, which has the advantage of correctly propagating error throughout the analysis, reducing the chance of false positive results and increasing power by disentangling potential correlations. We also carried out a two-step GWAS approach previously used on a smaller sample size of the Soays (Bérénos et al. 2015) to investigate whether any SNP-trait associations identified using our approach were due to the increased sample size or due to the different methodology (single-step vs. two-step GWAS).

Our aims were as follows:

1) To determine whether the increased density of SNPs changes the heritability estimates of the traits.

2) To determine whether the imputed SNP data enables the identification of new SNP-trait associations via GWAS.

3) To compare a single-step GWAS methodology with the two-step approach previously used on the study population.

Whilst we have used the Soay sheep as our study population, we believe that our objectives are also relevant for other wild populations. Keeping costs down is important for all research groups, and we aim to show that imputation is a way to do so whilst improving the power results of quantitative genetic analyses. We also intend to highlight the benefit of fitting fixed and random effects whilst performing GWAS instead of pre-correcting.

METHODS

Phenotypic data

The sheep are ear-tagged when they are first captured which allows for reidentification for life. We focused on five body size traits in three age groups: neonates, lambs, and adults. Of the five traits, three (weight, foreleg length and hindleg length) are live measures, recorded in April for neonates and in August for lambs and adults. The remaining two traits (metacarpal length and jaw length) are *post mortem* measures taken from skeletal material. Both birth and August weight are measured to the nearest 0.1kg, whilst the remaining traits are all measured to the nearest mm. A detailed description of trait measurements can be found in Beraldi et al. (2007).

We defined neonates as individuals who were caught and weighed between two and ten days after birth – birth weight was the only trait recorded for this age group. Lambs were classed as individuals who had phenotypic data recorded in the August of their birth year for the live traits, and as individuals who died before 14 months of age for the *post mortem* measures. Individuals were classed as adults if they had August phenotypic data recorded at least two years after birth, or if they died after 26 months of age for *post mortem* measures. We chose not to analyse yearling data due to the small sample sizes in comparison to the other age classes, which is due to high first winter mortality.

Genetic data

Most of the sheep in our study population have been genotyped using the Ovine SNP50 Illumina BeadChip, which targets 54,241 SNPs across the sheep genome. After removing SNPs which failed quality control standards (minor allele frequency (MAF) > 0.001, call rate > 0.99, deviation from Hardy-Weinberg Equilibrium P > 1e-05) and individuals with a call rate < 0.95, 39,368 polymorphic variants remained for 7630 individuals (3643 female, 3987 male). See Bérénos et al. (2014) for information on genetic sampling protocol and marker characteristics).

Of these 7630 individuals, 188 have also been genotyped using the Ovine Infinium HD SNP BeadChip which targets 606,066 SNPs. This has allowed for the low density genotypes to be imputed to the higher density using AlphaImpute, which combines shared haplotype and pedigree information for phasing and genotype imputation (Hickey et al. 2012) (see Stoffel et al. (2021) for information on imputation and quality control). We used imputed genotype "hard" calls (rather than genotype probabilities) in downstream analyses. After filtering SNPs that failed quality control standards, 419,281 autosomal SNPs remained for 7621 individuals (3639 females, 3982 males).

Both the 50K SNP data and the imputed SNP data are mapped to the OAR_v3.1 genome assembly.

Narrow sense heritability estimation

We used animal models to partition the phenotypic variance for each trait in each age class into genetic and non-genetic variance components. Fixed and random effects were fitted for all models, with the effects differing between traits and age classes (Table 1). We implemented these analyses in DISSECT (Canela-Xandri et al. 2015) using the following model:

$$y = X\beta + \sum_r Z_r u_r + Wg + \varepsilon$$

145 where y is the vector of phenotypic values; X is a design matrix linking individual records with the vector
146 of fixed effects β, Z_r is an incidence matrix that relates the random effect r to the individual records; u_r is
147 the associated vector of non-genetic random effects; g is the vector of additive genetic random effects
148 with W the incidence matrix; and ε is the vector of residuals. It is assumed that g ~ *MVN*(0, $M\sigma_g^2$), where
149 σ_g^2 is the additive genetic variance and M is the genomic relationship matrix (GRM). For each trait in
150 each age class, we ran this model twice: first with M being a GRM calculated from the 50K genotype
151 data, and second with M being a GRM calculated from the imputed SNP genotypes. The GRMs were
152 computed using DISSECT (Canela-Xandri et al. 2015) using VanRaden's Method 2 GRM calculation, for
153 which the genetic relationship between individuals i and j is computed as:

$$A_{ij} = \frac{1}{N} \sum_{k=1}^{N} \frac{(s_{ik} - 2p_k)(s_{jk} - 2p_k)}{2p_k(1 - p_k)}$$

154

155 where s_{ik} is the number of copies of the reference allele for SNP k of the individual i, p_k is the frequency
156 of the reference allele for the SNP k, and N is the number of SNPs (VanRaden 2008).

157 The narrow sense heritability was estimated by dividing the additive genetic variance (the variance
158 explained by the GRM) by the total estimated phenotypic variance (the sum of the variance explained by
159 the GRM and other fitted random effects after fitting fixed effects).

160 In adults, there are multiple records for August weight, foreleg length and hindleg length for the same
161 individual due to individuals being caught across multiple years. For these traits we used a repeatability
162 model in order that uncertainty was correctly propagated through all estimations (Mrode 2014). To
163 implement a repeatability model in DISSECT, we edited the input files so that each measurement had its
164 own row in the genotype and covariate files. Individual ID was replaced with a unique capture reference
165 number, and individual permanent environment was fitted as a random effect (see Supplementary
166 Methods for a more detailed explanation).

167 Sample sizes and total number of phenotypic measurements for all traits are shown in Table 1, with
168 effects fitted in all models.

169

170 *Genome wide association analysis*

171 Principal component analysis (PCA) using the GRM was performed prior to the genome-wide association
172 analyses (GWAS) using DISSECT (Canela-Xandri et al. 2015) in order to examine the underlying
173 population structure.

174 GWAS was also conducted using DISSECT (Canela-Xandri et al. 2015) using the following model:

$$y = X\beta + S\alpha + \sum_r Z_r u_r + Wg_{LOCO} + \varepsilon$$

175 where y, X, β, Z_r, u_r, W and ε are the same as in the model for heritability estimation, s is the effect of
176 the focal SNP, S is the design matrix linking individual records to the number of effect alleles for α, the
177 estimated SNP effect (coded as 0, 1 or 2), and gLOCO is the vector of additive genetic random effects
178 excluding the chromosome on which the focal SNP resides.

179 We fitted the same fixed and random effects for each trait and age class as for the heritability
180 estimation (Table 1). To account for population structure, when testing SNPs on a given chromosome for
181 association with the phenotype, a GRM calculated from the remaining autosomes (referred to as Leave
182 One Chromosome Out GRM (gLOCO) (Yang et al. 2014)) was fitted. Input files for repeated-measure
183 traits were reformatted as above. Our significance threshold was corrected for multiple testing using the
184 SimpleM method (Gao et al. 2008), which accounts for linkage disequilibrium between markers in order
185 to calculate the effective number of independent tests.

186 We estimated the variance explained by SNPs that passed the significance threshold using the equation

$$V(SNP) = 2pq\alpha^2$$

187 where p and q are the major and minor allele frequencies of the SNP, and α is the estimated SNP effect.
188 We then calculated the proportion of additive genetic variance explained by each SNP by dividing by the
189 total additive genetic variance estimated for that trait.

190 For any trait for which several SNPs in the same region were associated with variation in the trait and
191 thus had strong support for at least one QTL in the region, we carried out conditional analysis to
192 understand if the region could potentially harbour several independent QTL, or if further QTL could be
193 uncovered elsewhere in the genome. To that aim, the genotypes of the SNP with the smallest
194 association p value from each associated region (hereafter called the "top SNP") were added to the
195 GWAS model as a fixed covariate and removed from the GRMs and genotype data. The GWAS analysis
196 was re-run accounting for those associations to try and reveal novel peaks either in the same regions or
197 elsewhere in the genome.

198

199 *Genes in QTL regions*

200 For each trait x SNP association, we investigated the genes within a 0.5Mb window either side of the top
201 SNP to identify any genes which could be contributing to trait variation. We extracted a list of genes for
202 each trait using the biomaRt package in R (Durinck et al. 2005; Durinck et al. 2009) from the OAR_v3.1
203 genome assembly and reviewed each gene against the NCBI Gene (Bethesda (MD): National Library of
204 Medicine (US) 2004 - 2022) (including information from the Sheep Tissue Atlas (Jiang et al. 2014)),
205 Animal QTLdb (Hu et al. 2022), and Ensembl (Howe et al. 2020) databases to examine function and
206 expression annotations. When possible, we also compared with human and mouse orthologues due to
207 the high level of annotation data available for these two species.

208

209 *Two-step GWAS analysis*

GWAS has previously been performed on the adult traits in a smaller sample of Soay sheep using the 50K SNP data (Bérénos et al. 2015). The authors performed GWAS by first running a mixed model analysis, fitting fixed and random effects including whole-genome relatedness in the form of a GRM and, for repeated-measure traits, permanent environment. The residuals were then extracted and used as the phenotypic values for GWAS. For repeated measure traits, the mean residual value was used for each individual.

To investigate whether any novel SNP associations identified in this study were due to the increased sample size or due to the change in methodology, we also performed a two-step GWAS, focusing on adults only and using the 50K SNP data. Like Bérénos et al. (2015), we performed mixed model analyses using ASReml-R (Butler et al. 2017) and performed GWAS with the residuals as the trait phenotypes using DISSECT (Canela-Xandri et al. 2015). We used the Bonferroni correction calculated in Bérénos et al. (2015) to determine the significance threshold of 1.35e-6 for our two-step GWAS in order to compare with the previously published analysis.

225 RESULTS

226 *Heritability estimation*

227 Neonates

228 In neonates, the heritability of birth weight was 0.051 (S.E. 0.020) both when using the 50K SNPs to
229 calculate relatedness, and when using the imputed SNPs (Figure 1, Supplementary Table 1). Given that
230 both estimates are identical to 3 decimal places, there is no difference between the estimates.

231 Lambs

232 In lambs, the heritability estimates for the live August measures were lower than those for the *post*
233 *mortem* measures (Figure 1, Supplementary Table 1). Across all the traits, heritability estimates were
234 similar when using the 50K SNP data and the imputed SNP data, with the biggest difference being 0.024
235 for metacarpal length. For all traits, estimates were within one standard error of each other, indicating
236 that the small differences in heritability estimates between the two SNP densities were not significant.

237 Adults

238 As observed in lambs, heritability estimates for live measures in adults were lower than those of the
239 *post mortem* measures. Across all traits, heritability estimates were higher in adults than in lambs.
240 Estimates obtained using the 50K SNPs and using the imputed SNPs were similar and were within one
241 standard error of each other (Figure 1, Supplementary Table 1), meaning that the imputed SNPs
242 provided no additional information to partition the variation into genetic and environmental variance.

243 Estimates for all variance components are listed in Supplementary Table 1.

244

245 *GWAS*

246 *PCA*

247 The top 20 principal components together explained 10.68% of the variance in the genetic data, with the
248 top two principal components explaining 0.98% and 0.85% of the variance. We concluded that any
249 population structure that was likely to affect the GWAS results would be corrected for by fitting a LOCO
250 GRM in our GWAS model, as this will account for any structure caused by relationships.

251 *50K SNP data*

252 To correct for multiple testing, we calculated the effective number of tests to be 20082 using the
253 SimpleM method (Gao et al. 2008), giving a genome-wide significance threshold of $2.49e^{-06}$ for the 50K
254 SNP data.

255 For weight in neonates (birth weight), and lambs (August weight), no SNPs were found to have an
256 association p value smaller than this threshold, suggesting that any variants that influence weight
257 variation are either of small effect or were not tagged by SNPs in the 50K SNP data (Figure 2A,
258 Supplementary Figure 1B and 1G). For adult August weight, three SNPs had a p value lower than the
259 genome-wide significance threshold; one SNP on chromosome 6 and two SNPs on chromosome 9.

260 For all three leg length measures in lambs, we found associations with the same region on chromosome
261 16. SNP s23172.1 was the SNP with the lowest p value for lamb foreleg and hindleg, explaining 0.52%
262 and 0.69% of the genetic variance for each trait respectively (Supplementary Table 2, Supplementary
263 Figure 1C and 1D). For lamb metacarpal, SNP 22142.1 in the same chromosome 16 region had the
264 lowest p value and explained 0.97% of the genetic variance. There was also a single SNP on chromosome
265 3 (OAR3_100483326.1) and a cluster of SNPs on chromosome 19 that had p values smaller than the
266 genome-wide significance threshold and were associated with variation in lamb metacarpal length, with
267 the SNP with the lowest p value from each region explaining 2.08% and 2.40% of the genetic variance
268 respectively (Supplementary Table 2, Supplementary Figure 1E).

269 The two regions on chromosomes 16 and 19 that were associated with lamb metacarpal length variation
270 were also significantly associated with all three leg length measures in adults, with SNP s22142.1 on
271 chromosome 16 and SNP s74894.1 on chromosome 19 respectively explaining 0.80% and 2.04% of the
272 genetic variation in adult foreleg, 0.88% and 1.32% of the genetic variation in adult hindleg, and 0.55%
273 and 2.02% of the genetic variation in adult metacarpal length. There were other regions of the genome
274 also associated with variation in the adult leg length traits; a region on chromosome 11 was significant
275 across all three adult leg length traits, with the most significant SNP explaining 2.35%, 2.25% and 1.13%
276 of the genetic variance in adult foreleg, hindleg and metacarpal respectively (Figure 2B, Supplementary
277 Table 2, Supplementary Figure 1H and 1J). For adult foreleg, a SNP on chromosome 7 and two on
278 chromosome 9 were also associated, with the most significant SNPs in each region explaining 1.31% and
279 2.99% of the genetic variance respectively for this trait (Supplementary Table 2, Supplementary Figure
280 1H).

281 In lambs, there were no associations with jaw length found (Supplementary Figure 1F). In adults, a SNP
282 on chromosome 20 was associated with jaw length variation, explaining 2.05% of the genetic variance
283 for this trait (Supplementary Table 2, Supplementary Figure 1K).

284 In total, we identified 85 SNP-trait associations with 39 unique SNPs.

285 *Imputed data*

286 Using the SimpleM method (Gao et al. 2008), we calculated the number of effective tests to be 48635,
287 giving a genome-wide significance threshold of $1.03e^{-06}$.

288 When performing GWAS using the imputed SNP data, we were able to recover significant SNPs in the
289 same locations for all traits as those we found using the 50K SNP data. Of the 85 SNP-trait associations
290 that we identified with the 50K SNP data, 81 were significant using the imputed SNP data – the
291 remaining four SNPs were no longer significant due to the increased multiple testing burden (which
292 leads to a more stringent significance threshold) between the 50K SNP data and the imputed SNP data
293 ($2.49e^{-06}$ and $1.03e^{-06}$ respectively).

294 We also identified 795 new SNP-trait associations using the imputed SNP data with 425 unique SNPs
295 (Supplementary Table 2). The majority of new associations were in the same regions as the SNPs
296 identified using the 50K SNP data, but we also found new associations: four SNPs on chromosome 1 and
297 three SNP on chromosome 7 was associated with birth weight (Figure 2A, Supplementary Table 2), one
298 SNP on chromosome 3 was associated with adult August weight (Figure 2B, Supplementary Table 2), and

one SNP on chromosome 17 was associated with adult metacarpal length (Figure 2C, Supplementary Table 2).

Manhattan and QQ plots for all traits can be found in Supplementary Figure 1.

Conditional analysis

We performed conditional analysis on all three leg length traits in both lambs and adults, as well as on birth weight, adult August weight adult jaw length (See Supplementary Table 2 for all SNPs that were fitted for each trait). For all of these traits we performed the conditional analysis using both the 50K SNP data and the imputed SNP data, with the exception of birth weight, which did not have any significant SNP associations using the 50K data.

Six of the nine traits we performed conditional analysis on had significant SNPs after fitting the SNPs with the lowest p value, however for four of these traits (lamb metacarpal length, adult August weight, foreleg length and hindleg length), these were SNPs that were also significant in our original GWAS analysis but were not fitted in the conditional analysis due to being the only SNP that was significantly associated with the trait in that region (Supplementary Table 3). The remaining two traits (birth weight and adult jaw length) both had a new association, both of which were on chromosome 2. For birth weight, nine SNPs had p values lower than the genome-wide significance threshold, all around ~81Mb (Figure 3A, Supplementary Table 3). For adult jaw length, only one SNP had a lower p value than the genome-wide significance threshold, at position 137,162,126 (Figure 3B, Supplementary Table 3).

Genes in QTL regions

Given that all of the region-trait associations that were found to be significant with the 50K SNP data were also significant with the imputed SNP data, we chose to focus on top SNPs in the imputed dataset, including the two regions that were only significantly associated when performing the conditional analysis (See Supplementary Table 2 and 3 for the list of SNPs, and Supplementary Table 4 for the list of genes).

We found a total of 179 genes in the regions around the SNPs associated with our traits. 56 of these genes were unannotated in the current sheep genome build, and of those that were annotated, three did not have a listed mouse homologue and a further six had neither a mouse nor a human homologue. Of the genes that did have annotation and homologue data, we found nine that are associated with similar traits to our focal traits in humans and mice, suggesting that they may be contributing to the genetic variation of our traits (Table 2). However, without intimate knowledge of the genes surrounding the focal SNPs, it is likely that there are other genes that are also contributing. It is also worth noting that variation in coding regions of genes in proximity to SNPs we identified as being associated with our traits may not be responsible for variation in the phenotypes, but instead the causal variants may lie in regulatory sequences that modulate expression of either these or other genes.

We also compared our GWAS results with QTL from Animal QTLdb (Hu et al. 2022). We found that the region on chromosome 6 that we found to be associated with adult August weight overlaps with a region previously found to be associated with carcass weight and final body weight in an (Awassi x Merino) x Merino backcross population (Cavanagh et al. 2010) and is ~0.5Mb upstream of a 2.5Mb

338 region that has also previously been associated with body weight in a population of Australian Merino
339 sheep (Al-Mamun et al. 2015). In addition, the region on chromosome 9 we found to be associated with
340 adult August weight is 1Mb upstream of a region previously found to be associated with yearling live
341 weight in a population of Chinese Merino sheep.

342 Chromosome 6 has previously been associated with adult body weight using linkage analysis in a smaller
343 sample of Soays (Beraldi et al. 2007), however the microsatellite markers flanking the associated region
344 are not located close to the region we identified.

345

346 *Two-step GWAS*

347 Across all 5 traits when using the two-step approach, we recovered the SNP-trait associations identified
348 by Bérénos et al. (2015). However, we were unable to recover any of the novel SNP-trait associations we
349 found when performing our single-step GWAS on the 50K SNP data, with the exception of the
350 association between chromosome 16 and adult foreleg (though Bérénos et al. noted that SNPs in this
351 region approached significance in their analysis). Despite the genome-wide significance threshold used
352 by Bérénos et al. (2015) being more stringent than the significance threshold we calculated using the
353 SimpleM method for the 50K SNP data, no additional associations are recovered when using our less
354 stringent threshold

355 Our QQ plots using the two-step method also matched the QQ plots of Bérénos et al. (2015). In both,
356 the observed p values were higher than the expected p values, causing the majority of points in the
357 plots to fall below the x=y line (Supplementary Figure 3).

DISCUSSION

Heritability

Our results corroborate findings from previous studies in the Soay sheep. For instance, we showed that all five body size traits we studied in Soay sheep are influenced by genetic variation in the population (Bérénos et al. 2014), and that *post mortem* measures have higher heritability estimates than live measures . We also found that leg measures have higher heritability than weight (Wilson et al. 2006; Beraldi et al. 2007; Bérénos et al. 2014), and that heritability estimates increase with age (Wilson et al. 2006; Bérénos et al. 2014).

The heritability estimates for the 50K data were very similar to those estimated using a GRM based on the 50K data in a smaller sample of the same population of sheep by Bérénos et al. (2014), with estimates for the same trait falling within one standard error of each other. The biggest difference was in adult metacarpal length with a heritability difference of 0.05 (estimates were 0.644 (0.047) and 0.594 (0.047) for our and Bérénos et al.'s results respectively). Given that we used the same models as Bérénos et al., it is likely that the small differences between heritability estimates for each trait is due to our increased sample sizes.

Comparing the heritabilities estimated using the imputed SNP data against the estimates using the 50K SNP data, we found little difference between the two SNP densities in any traits in any age class. The additional genotypes at the imputed SNPs do not give any additional information on additive genetic variation for these traits. This result is not surprising given the previous rarefaction analysis showing that the heritability of these body size traits in adults asymptoted when about half the 50K SNP data was used (Bérénos et al. 2014). There is high LD between nearby SNPs in the Soay sheep genome, which suggests that most, if not all, of the causal variants tagged by the imputed SNP data may have already been tagged by the 50K SNPs. The high LD was reflected when calculating GWAS significance thresholds – whilst the number of SNPs between the 50K SNP data and the imputed SNP data increased by a factor of ten, the number of effective tests only doubled (39K SNPs, 20082 effective tests and 401K SNPs and 48635 effective tests respectively).

For some of the traits we have analysed there is still a difference in heritability estimated using SNP data versus heritability estimated using pedigree – for example, the highest SNP-based heritability estimate for lamb metacarpal length (the estimate using the imputed SNP data) gave an estimate 59% of Bérénos et al.'s pedigree-based estimate (Bérénos et al. 2014). Given that our SNP-based heritability estimates were similar when using the 50K SNP data as when using the imputed SNP data, and the results of Bérénos et al.'s rarefaction analysis (Bérénos et al. 2014), we believe it is unlikely that increasing the density of genotyped SNPs that are common in the population will increase heritability estimates of these traits. It is possible instead that the difference in heritability estimates obtained from pedigree and genomic data is due to rare familial variants that do not segregate widely in the population, as well as due to dominance and epistasis.

GWAS

Body size traits have been the focus of many kinds of analyses in Soay sheep (Beraldi et al. 2007; Ozgul et al. 2009; Bérénos et al. 2014; Bérénos et al. 2015; Pemberton et al. 2017; Regan et al. 2017; Ashraf et

398 al. 2021), and several SNP associations have already been identified for these traits. A 2015 study aiming
399 to find SNP-trait associations identified QTL for adult leg length measures on chromosomes 16 and 19
400 (s23172.1 and s74894.1 respectively) (Bérénos et al. 2015). A more recent study comparing genomic
401 prediction methods in Soays using the 50K SNP data identified s48811.1 on chromosome 7 and s50107.1
402 on chromosome 9 as having a probability higher than 0.9 of having a non-zero effect on adult foreleg
403 length in addition to the previously discovered regions on chromosomes 16 and 19 (Ashraf et al. 2021).
404 We were able to identify all four of these associations in our GWAS, alongside associations that have not
405 previously been identified in this population. Use of the imputed SNP data allowed us to discover four
406 more associations with loci that were not genotyped in the 50K SNP data, indicating that future
407 identification of polymorphisms influencing trait variation in the Soay sheep will benefit from using the
408 imputed data.

409 Performing a two-step analysis confirmed that the novel SNP-trait associations we were able to identify
410 using the 50K SNP data were due to fitting the fixed and random effects for each trait simultaneously,
411 performing GWAS in a single step, rather than due to having a larger sample size. Given the increase in
412 SNP-trait associations when using the single-step methodology, and with the availability of software like
413 DISSECT which is able to fit fixed and random effects whilst performing GWAS, two-step GWAS should
414 be avoided. As we have shown, although DISSECT does not currently have the option to automatically
415 run a repeated measures GWAS, it is possible to modify input files to allow for repeated measures to be
416 appropriately modelled.

417 The imputed SNP data revealed SNP-trait associations in four regions of the genome that were not
418 discovered using the 50K SNP data; a region on chromosome 1 and a region on chromosome 7 and birth
419 weight, a region on chromosome 3 and adult August weight, and a region on chromosome 17 and adult
420 metacarpal length. (Supplementary Table 2). When examining the Manhattan plot for the 50K data for
421 each trait (Figure 2A, 2B and 2C, Supplementary Table 2) it is clear that, with the exception of the region
422 on chromosome 1 associated with birth weight, there was a small cluster of SNPs just under the
423 significance threshold in the 50K analyses. The additional (imputed) SNPs may have matched the allele
424 frequency of the underlying causal variants more accurately, resulting in a smaller association p value.

425 We performed conditional analysis on all three leg length traits in both lambs and adults, as well as on
426 birth weight (only using the imputed SNP data), adult August weight and adult jaw length. For each trait,
427 we simultaneously fitted the genotype for the SNP with the lowest p value for any chromosome that had
428 at least two SNPs found to be associated with the trait (see Supplementary Table 2 for a list of SNPs
429 fitted for each trait). We found that all of the SNPs that were significant in the GWAS analysis were no
430 longer significant in the conditional analysis when a significant SNP on the same chromosome was fitted
431 (Figure 3, Supplementary Table 3). We suggest that any future work looking to pinpoint the exact
432 location of the genetic variants affecting body size traits in Soay sheep primarily focus on the regions
433 around the SNPs listed in Supplementary Table 2.

434 We identified 179 genes within 0.5Mb of the top SNPs for each trait (Supplementary Table 4), and of
435 these genes, we found nine that are potential candidate genes for further analyses due to their
436 association with similar traits in other species. Two of these genes (*CYP26B1* and *EXOC6B*) are associated
437 with the same trait (lamb metacarpal length) and are located within 0.5Mb of the same top SNP on
438 chromosome 3. *CYP26B1* is associated with skeletal abnormalities in humans and zebrafish (Laue et al.
439 2011) and *CYP26B1* knockouts produced reduced limbs in mice (Yashiro et al. 2004), whilst *EXOC6B* is

440 associated with spondyloepimetaphyseal dysplasia in humans which symptoms include skeletal
441 malformations affecting the long bones of the limbs and short stature (Campos-Xavier et al. 2018).

442 *PTH1R* is located in the region on chromosome 19 associated with lamb metacarpal length and all three
443 adult leg traits, and has been found to be involved in osteoblast development in mice (Qiu et al. 2015),
444 and is associated with skeletal disorders such as Eiken's syndrome (Duchatelet et al. 2005), Jansen's
445 metaphyseal chondrodysplasia and Blomstrand's lethal chondrodysplasia (Schipani and Provot 2003). As
446 the top SNPs on chromosome 19 for both lamb and adult metacarpal are in a slightly different position
447 to the top SNP for the live measures, *LTF* is in the region associated with both lamb and adult
448 metacarpal length on chromosome 19, and has been found to increase bone growth when injected into
449 piglets (Li et al. 2018), stimulate osteoblast proliferation in humans (Cornish and Naot 2010), and has
450 high expression levels in human bone marrow (Fagerberg et al. 2014).

451 *BMP6*, located in the region on chromosome 20 associated with adult jaw length, has also been found to
452 be involved in bone development and expression in mice jaw bone (Oralová et al. 2014).

453 We found three potential candidate genes associated with weight. *FAM13A* in the region on
454 chromosome 6 associated with adult August weight has been found to modulate body fat distribution
455 and adipocyte function in humans and mice (Fathzadeh et al. 2020), affects adipose insulin signalling in
456 mice (Wardhana et al. 2018), and is associated with obesity also in mice (Tang et al. 2019). *PENK*, located
457 in the region on chromosome 9 associated with adult August weight has been found to cause diminished
458 food motivation, lower baseline body weight and attenuated weight gain when knocked out in mice
459 (Mendez et al. 2015). *ONECUT1*, located in the region on chromosome 7 associated with birth weight
460 has been found to be associated with birth weight in humans (Warrington et al. 2019). Unlike the other
461 potential candidate gene-trait associations we found, this association was only found when performing
462 GWAS with the imputed SNP data.

463 Although we have discovered new SNP-trait associations, it is likely that there are still causative variants
464 that remain undetected. GWAS lacks power to detect rare causative variants and variants with very
465 small effect sizes (Yang et al. 2010). Also, GWAS power drops when the same amount of phenotypic
466 variation is a consequence of multiple variants in the same region as opposed to a single variant
467 (Nagamine et al. 2012). Regional mapping methods have been developed that partition trait variance
468 into regions by simultaneously fitting a whole genome and a regional GRM, with the regions either being
469 defined as fixed SNP windows (Nagamine et al. 2012) or haplotype blocks (Shirali et al. 2018). Such
470 methodologies have the potential to identify regions of the genome that contain variants associated
471 with a trait that are unable to be identified by GWAS either due to being rare, or individually having
472 small effects on trait variation. Genomic prediction, which simultaneously estimates all marker effects
473 drawn from multiple distributions, can also be used to study the genetic architecture of traits by
474 estimating the posterior inclusion probability of a SNP having a non-zero effect on a trait. Genomic
475 prediction has already been used on adult body size traits in Soays, and has identified several of the
476 SNPs we identified through our GWAS approach (Ashraf et al. 2021). Ultimately, we believe that it is
477 important to use a variety of methodologies when studying the genetic architecture of complex traits, as
478 different analyses have different strengths and may be able to identify different QTL.Across all traits for
479 all age classes, the QQ plots showed deviation from the expected distribution of test statistics under the
480 null hypothesis (x=y line) for a wide range of test statistics, including low values, which may be either
481 due to underlying population structure not accounted for by the GRMs or due to trait architecture. The

482 first 20 genomic principal components accounted for only 10.68% of the variance in the genetic data,
483 and repeating the GWAS analysis fitting these first 20 genomic principal components in addition to the
484 GRM did not change the p values of the SNPs nor the QQ plots (data not shown). This shows that the
485 principal components in this case were not useful in adjusting for population structure in the presence
486 of the GRM, and that population structure is not likely to cause any issues in our analyses, especially
487 after fitting a LOCO GRM.

488 In order to have sufficient power to detect associations between markers and a trait of interest, GWAS
489 primarily requires two factors: i) a very high density of genotyped SNPs, and ii) a large number of
490 individuals that have been genotyped and phenotyped (Santure and Garant 2018). For intensively
491 studied organisms, both are achievable; such populations tend to have more individuals accessible to
492 collect data from, high density genotyping can be done at a lower cost due to higher demand, and, as in
493 humans, data from different populations can be combined to create larger sample sizes. GWA studies of
494 humans are the most obvious example of this; studies often have study populations made up of
495 hundreds of thousands of individuals and human SNP chips commonly genotype hundreds of thousands
496 of variants (for example, see Wood et al. 2014; Ishigaki et al. 2020; Wu et al. 2021). In comparison, wild
497 study population samples are much smaller – often struggling to reach one thousand individuals – and
498 the number of SNPs genotyped is much lower (for example, see Silva et al. 2017; Malenfant et al. 2018;
499 Perrier et al. 2018). Analyses of wild populations therefore generally lack the power of more intensively
500 studied study organisms. Here, we have increased power by increasing the number of genotyped
501 markers via imputation. Despite high LD in the Soay sheep population, use of imputed data has allowed
502 us to identify four new SNP-trait associations, including an association with birth weight, which had yet
503 to be associated with any QTL in the Soay population. We have therefore shown that for a given sample
504 size, more information can be obtained by increasing the density of markers for those individuals have
505 been phenotyped. We suggest that, where possible, analyses of wild populations impute SNP data in
506 order to increase power and obtain results that may otherwise remain undiscovered.

507 In our population, we were able to identify new SNP-trait associations when performing GWAS with the
508 higher density imputed dataset that were not found with the lower density non-imputed data – one of
509 which allowed for the identification of a potential candidate gene for that trait. We have shown that
510 analyses performed with imputed data, such as heritability estimation and GWAS, can still pick up
511 additive genetic variation that would have been identified with the non-imputed data. We suggest that,
512 where possible, analyses of wild populations impute SNP data in order to increase power and obtain
513 associations that may otherwise remain undiscovered. In addition, we demonstrated the benefits of
514 fitting fixed and random effects during GWAS instead of pre-correcting and performing GWAS on the
515 residuals. We therefore recommend that researchers use this "one-step" method when performing
516 GWAS on wild populations.

517

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675

Age	Trait	No. individuals	No. records	Fixed effects	Random effects
Neonate	Birth weight	2678	2678	Sex	Year of birth
				Litter size	Mother ID
				Population size year before birth	
				Age of mother (quadratic)	
				Ordinal date of birth	
				Age (days)	
Lamb	Weight	2228	2228	Sex	Year of birth
				Litter size	Mother ID
				Population size	Permanent environment
				Age (days)	
	Foreleg	2284	2284	Sex	Year of birth
				Litter size	Mother ID
				Population size	Permanent environment
				Age (days)	
	Hindleg	2349	2349	Sex	Year of birth
				Litter size	Mother ID
				Population size	Permanent environment
				Age (days)	
	Metacarpal	2059	2059	Sex	Year of birth
				Litter size	Mother ID
				Age at death (months)	
	Jaw	2113	2113	Sex	Year of birth
				Litter size	Mother ID
				Age at death (months)	
Adult	Weight	1152	3553	Sex	Year of capture
				Population size	Permanent environment
				Age (years)	
	Foreleg	1121	3331	Sex	Year of capture
				Population size	Permanent environment
				Age (years)	
	Hindleg	1135	3444	Sex	Year of capture
				Population size	Permanent environment
				Age (years)	
	Metacarpal	945	945	Sex	Year of birth
				Age at death (years)	
	Jaw	991	991	Sex	Year of birth
				Age at death (years)	

Table 1 Number of individuals and records, fixed and random effects fitted in each trait x age class model in addition to the GRM. The same individuals and records were used for both heritability estimates and for GWAS.

Gene Name	Ensembl Gene ID	Chr	Associated trait	Effects in other species
Cytochrome P450 26B1	ENSOARG00000011582	3	Lamb metacarpal length	Associated with skeletal abnormalities in humans and zebrafish (Laue et al. 2011), knockouts produce reduced limbs in mice (Yashiro et al. 2004).
EXOC6B	ENSOARG00000011607	3	Lamb metacarpal length	Associated with spondyloepimetaphyseal dysplasia (resulting in short stature) in humans (Campos-Xavier et al. 2018).
FAM13A	ENSOARG00000018727	6	Adult August weight	Modulates body fat distribution and adipocyte function in humans and mice (Fathzadeh et al. 2020) as well as adipose insulin signalling in mice (Wardhana et al. 2018), also linked with obesity in mice (Tang et al. 2019)
ONECUT1	ENSOARG00000020928	7	Birth weight	Associated with birth weight in humans (Warrington et al. 2019).
IFT43	ENSOARG00000002065	7	Adult foreleg length	Associated with Sensenbrenner syndrome (resulting in growth retardation and dwarfism due to femoral and humeral limb shortening) in humans (Arts et al. 2011).
PENK	ENSOARG00000020184	9	Adult August weight	PENK knock-out mice found to have diminished food motivation, lower baseline body weight and attenuated weight gain (Mendez et al. 2015)
PTH1R	ENSOARG00000006638	19	Lamb metacarpal length, adult foreleg length, adult hindleg length, adult metacarpal length	Involved in osteoblast development in mice (Qiu et al. 2015), associated with skeletal disorders such as EKNS (Duchatelet et al. 2005), JMC and BLC (Schipani and Provot 2003) in humans.
LTF	ENSOARG00000008620	19	Lamb metacarpal length, adult metacarpal length	Human LTF associated with increased bone growth when injected into piglets (Li et al. 2018), found to stimulate osteoblast

				proliferation (Cornish and Naot 2010). High expression levels in human bone marrow (Fagerberg et al. 2014).
BMP6	ENSOARG00000017264	20	Adult jaw length	Involved in bone development and expressed in the jaw bone in mice (Oralová et al. 2014).

680 **Table 2** Potential candidate genes for future analyses. From left to right: gene name, Ensembl gene ID,
681 chromosome, associated trait, and evidence for association in other species.

682

683 **Figure 1** Estimates of heritability for body size traits in neonates, lambs, and adult Soay sheep when
684 using a GRM calculated from the 50K SNP data (blue) compared with using a GRM calculated from the
685 imputed SNP data (yellow). Error bars represent standard error estimates.

686

687

688 **Figure 2** Manhattan plots for A) birth weight GWAS using 50K SNP data (left) and imputed SNP data
689 (right); B) adult August weight GWAS using 50K SNP data (left) and imputed SNP data (right); and C)
690 adult metacarpal length GWAS using 50K SNP data (left) and imputed SNP data (right). The red line
691 represents the significance threshold ($2.49e^{-06}$ for the 50K SNP data and $1.03e^{-06}$ for the imputed SNP
692 data) – any SNPs above this threshold are considered to be significantly associated with variation in their
693 respective traits.

694

695

696 **Figure 3** Miami plots for A) birth weight using imputed SNP data (top) and birth weight conditional
697 analysis using imputed SNP data (bottom); and B) adult jaw length GWAS using 50K SNP data (top left)
698 and imputed SNP data (top right), adult jaw length conditional analysis using 50K SNP data (bottom left)
699 and imputed SNP data (bottom right). The red line represents the significance threshold ($2.49e^{-06}$ for the
700 50K SNP data and $1.03e^{-06}$ for the imputed SNP data) – any SNPs above this threshold are considered to
701 be significantly associated with variation in their respective traits.

702

Data Availability Statement

All scripts and data can be found at
https://github.com/CaelinnJames/Impact_of_SNPDensity_on_Soay_Sheep

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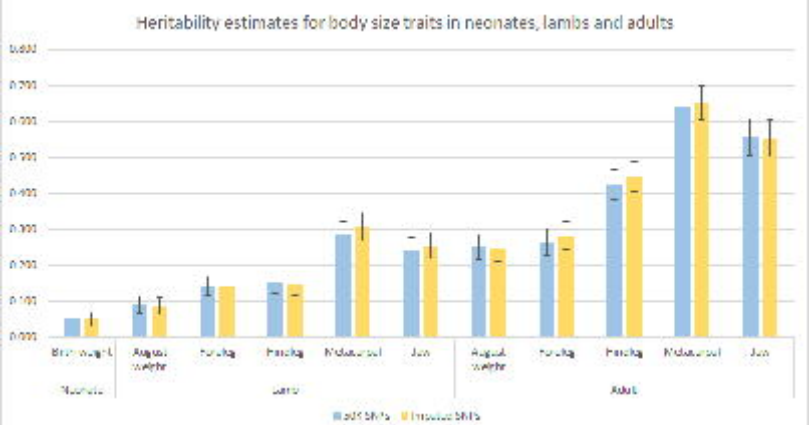
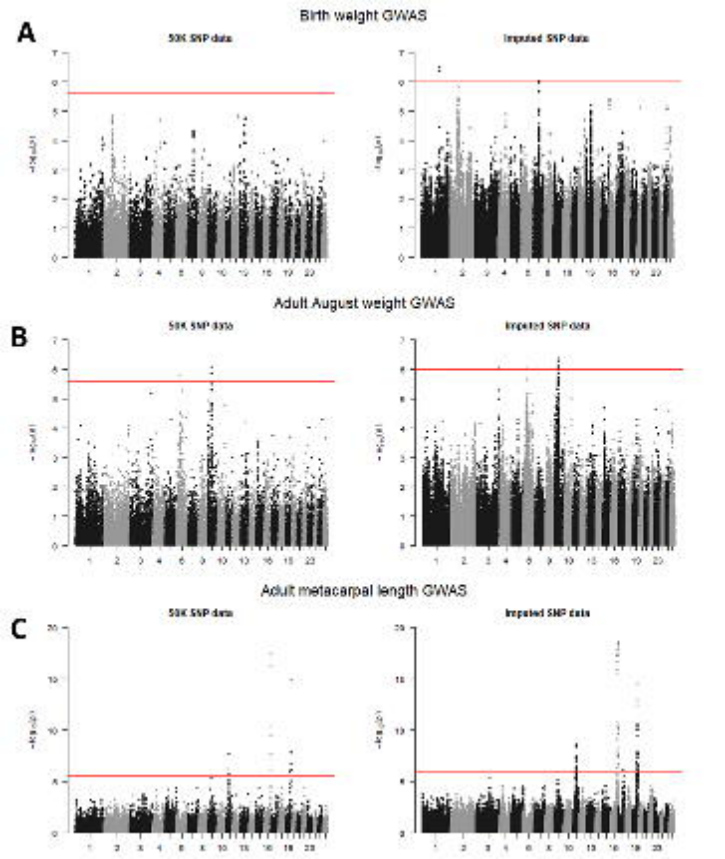
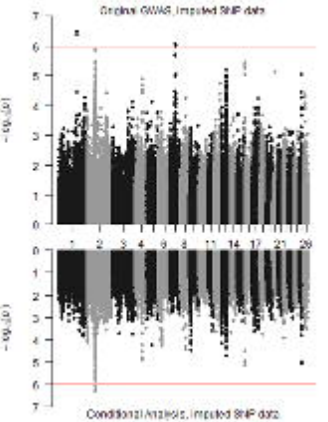
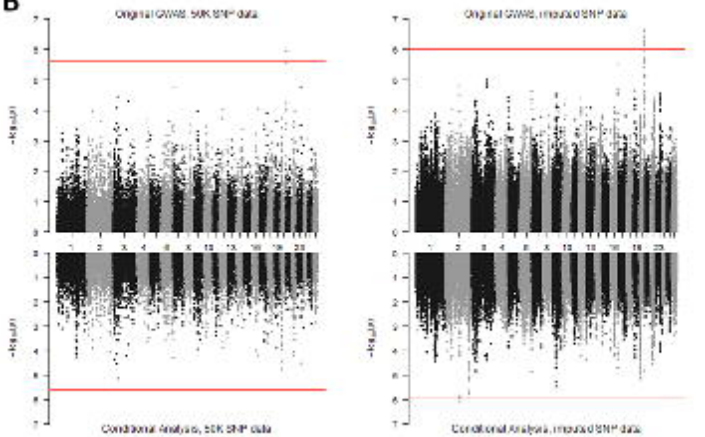

Heritability estimates for body size traits in neonates, lambs and adults


A
Birth weight GWAS
50K SNP data
Imputed SNP data
B
Adult August weight GWAS
50K SNP data
Imputed SNP data
C
Adult metacarpal length GWAS
50K SNP data
Imputed SNP data

A

Birth weight conditional GWAS analysis



Adult jaw length conditional GWAS analysis

B