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). 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 five new SNP-trait associations that were not found with the lower density dataset. Conditional GWAS analyses after fitting the most significant SNPs revealed two more novel SNP-trait associations.

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 (Barton and Keightley 2002), and understand micro-evolutionary dynamics that occur due to environmental change. 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.

A popular method to decompose phenotypic variation in wild populations into genetic variance and environmental variance is the 'animal model', originally developed by animal breeders (Henderson 1984; Kruuk 2004; Wilson et al. 2010). As part of the model, genetic relatedness is fitted, which is often derived from a pedigree. Pedigrees can be constructed using field observations, assigning parentage using genetic markers, or a mixture of both (Pemberton 2008). However, wild pedigrees are often short, incomplete, and contain errors: observational data may be inaccurate due to incorrect parent-offspring assumptions, and if the genetic markers chosen are not sufficiently discriminatory they may result in misassigned parentage. Erroneous pedigree links can bias results of analyses using animal models; for example, misidentification of sires in cattle resulted in decreased heritability estimates for milk yield, fat yield, and milk-fat ratio (Van Vleck 1970).

In place of a pedigree, genotypes at multiple polymorphic loci can be used to estimate relatedness for use in an animal model. This has the advantage of not relying on recovering a pedigree and thus is not affected by incomplete or incorrect familial links (though knowledge of pedigree relationships is still valuable, for example so that maternal effects can be fitted). Relatedness estimated using genotype data is also potentially more precise than that from pedigrees – for example, with a pedigree it is presumed that full-sibs have a relatedness of 0.5, however the exact relationship varies depending on which DNA segments each sib has inherited (Visscher et al. 2006). Despite this greater accuracy, genotype-based relatedness estimates can still vary depending on which variants in the population have been genotyped, and the density of the genotyped variants. Increasing the density of genotyped polymorphisms means they are more likely to be in linkage disequilibrium (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. Thus, in species such as humans where genotyping is commonly of unrelated individuals and LD is generally low, the estimated heritability of a trait increases with SNP density due to an increase in the number of causal variants being in LD with genotyped SNPs (for instance, heritability of human height was estimated to be 0.45 when using 294,831 SNPs (Yang et

al. 2010) and 0.56 when using ~17 million imputed SNPs (Yang et al. 2015)). However, increasing the number of genotyped markers means larger, denser genotyping arrays with prices 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, life history and environmental data is 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.

To date, quantitative genetic analyses of the Soays have been performed using either the pedigree or the 50K SNP data. Bérénos et al. (2014) investigated the difference in quantitative genetic parameter estimates when using the pedigree or a genomic relationship matrix (GRM) constructed from the 50K SNP data. The authors estimated heritability, maternal genetic effects and genetic correlations for body size traits (weight, foreleg length, hindleg length, metacarpal length and jaw length) across four age groups. The additive genetic variance and the heritability estimates using the GRM were lower than when using the pedigree to estimate relatedness, with the SNPs explaining 84% of the additive genetic variance of the pedigree on average, though for the majority of the traits the standard errors of the pedigree-based and SNP-based heritability estimates were within one standard error of each other. Genetic correlations were found to differ little between analyses using the different relatedness estimates. A SNP rarefaction analysis of the heritability estimates was conducted on the adult traits, and it was found that heritability estimates asymptoted when 50% of the SNPs were used to estimate relatedness.

Linkage disequilibrium is high in Soay sheep (Bérénos et al. 2014), which may explain the results of the rarefaction analysis: the same causal variations can be represented by multiple genotyped SNPs, and so many genotyped SNPs are not providing unique information towards the additive genetic variance. However, given that heritability estimates when using SNP-based estimates of relatedness were lower than when using pedigree-based estimates, it is also possible that there are variants that contribute to variation in these traits that aren't in perfect LD with the genotyped SNPs. This may be depressing estimates of additive genetic variance in comparison to pedigree-based estimates. Increasing the

density of genotyped SNPs may therefore increase the SNP-based heritability estimates of these traits to closer match the pedigree-based estimates.

Bérénos et al. (2015) focused on the same traits as Bérénos et al. (2014) in adults only, and partitioned the genetic variance for each trait, first by chromosome and then by 150 SNP windows, and performed GWAS. For the three leg length phenotypes, a disproportionately high amount of variance was explained by two SNPs on chromosome 16 (s23172.1) and chromosome 19 (s74894.1). For the remaining traits, the proportion of additive genetic variance explained by a chromosome was proportional to its length, suggesting that variation in these traits is influenced by many genetic variants of small effect, and no SNP-trait associations were discovered. The authors performed a two-step GWAS analysis; first they modelled the traits by fitting fixed and random effects, and then they extracted the residuals from the models and tested for association between SNPs and the residuals. Three traits (weight, foreleg length and hindleg length) are measured in the same individual across multiple years – for these traits, the authors analysed the mean residual values.

Since the studies from 2014 and 2015, 1895 more individuals have been both phenotyped and genotyped on the Ovine SNP50 Illumina Beadchip. In addition, 188 individuals were genotyped on the Ovine Infinium High Density chip with 600K attempted SNPs, which has enabled the genotypes of the remaining individuals to be accurately imputed to that higher density using LD and pedigree data (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 using an increased sample size and compared the results to the previous studies (Bérénos et al. 2014; Bérénos et al. 2015). We focused on the same five traits as the previous studies in neonates, lambs and adults. Unlike the 2015 study (Bérénos et al. 2015), we performed GWAS by fitting fixed and random effects in the same step as testing for SNP-trait associations. This has the advantage of correctly propagating error throughout the analysis, reducing the chance of false positive results. We also carried out a two-step GWAS approach similar to that of Bérénos et al. 2015, focusing on the adult traits using the 50K SNP data, to investigate whether any SNP-trait associations identified using our approach were due to the increased population 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 using the same individuals for both the low SNP density and the high SNP density analysis. Given the previous rarefaction analysis (Bérénos et al. 2014), our prediction was that there would be no change.

2) To determine whether the imputed SNP data enables the identification of new SNP-trait associations via GWAS. We predicted we would find more associations, either due to increased power to detect small effect size SNPs through increased LD with the imputed SNPs or due to tagging of new causal variants.

3) To examine how a single-step GWAS methodology compares with the two-step approach previously used on the Soay population, to investigate whether any novel SNP-trait associations

155 identified with the 50K SNP data were due to increased population size or due to the difference
156 in methods.

METHODS

Phenotypic data

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. The sheep are ear-tagged when they are first captured which allows for reidentification for life. 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. Unlike Bérénos et al. (2014), 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), 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 types 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). 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:

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

195 where y is the vector of phenotypic values; X is a design matrix linking individual records with the vector
196 of fixed effects β, Z_r is an incidence matrix that relates a random effect to the individual records; u_r is the
197 associated vector of non-genetic random effects; g is the vector of additive genetic random effects with
198 W the incidence matrix; and ε is the vector of residuals. It is assumed that g ~ *MVN*(0, $M\sigma_g^2$), where σ_g^2 is
199 the additive genetic variance and M is the genomic relationship matrix (GRM). For each trait in each age
200 class, we ran this model twice: first with M being a GRM calculated from the 50K genotype data, and
201 second with M being a GRM calculated from the imputed SNP genotypes. The GRMs (VanRaden 2008)
202 were computed using DISSECT (Canela-Xandri et al. 2015), and the genetic relationship between
203 individuals i and j is computed as:

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

204

205 where s_{ik} is the number of copies of the reference allele for SNP k of the individual i, p_k is the frequency
206 of the reference allele for the SNP k, and N is the number of SNPs.

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

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

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

219

220 *Genome wide association analysis*

221 We also conducted genome-wide association analyses using DISSECT (Canela-Xandri et al. 2015). We
222 fitted the same fixed and random effects for each trait and age class as for the heritability estimation
223 (Table 1). To account for population structure, when testing SNPs on a given chromosome for
224 association with the phenotype, a GRM calculated from the remaining autosomes (referred to as Leave
225 One Chromosome Out GRM (Yang et al. 2014)) was fitted. Input files for repeated-measure traits were
226 reformatted as above. Our significance threshold was corrected for multiple testing using the SimpleM

227 method (Gao et al. 2008), which accounts for linkage disequilibrium between markers in order to
228 calculate the effective number of independent tests.

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

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

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

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

241

242 *Genes in QTL regions*

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

250

251 *Two-step GWAS analysis*

252 To investigate whether any novel SNP associations identified (since Bérénos et al. 2015) by performing
253 GWAS on the adult traits using the 50K SNP data were due to the increased population sample or due to
254 the change in methodology, we also performed a two-step GWAS, focusing on adults only and using the
255 50K SNP data. We performed mixed model analyses using ASReml-R (Butler et al. 2017) for each trait
256 fitting the same fixed and random effects as in our single-step analyses, including whole-genome
257 relatedness (in the form of a GRM) and, for repeated-measure traits, permanent environment. We then
258 extracted the residuals from the mixed models and performed GWAS with the residuals as the trait
259 phenotypes using DISSECT (Canela-Xandri et al. 2015). For repeated measure traits, we used the mean
260 residual value for each individual. We used the Bonferroni correction calculated in Bérénos et al. 2015 to
261 determine the significance threshold for our two-step GWAS in order to best compare with previous
262 GWAS performed on the Soay sheep.

263

264 RESULTS

265 *Heritability estimation*

266 Neonates

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

270 Lambs

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

276 Adults

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

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

283

284 *GWAS*

285 *50K SNP data*

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

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

294 For all three leg length measures in lambs, we found associations with the same region on chromosome
295 16. SNP s23172.1 was the SNP with the lowest p value for lamb foreleg and hindleg, explaining 0.52%
296 and 0.69% of the genetic variance for each trait respectively (Supplementary Table 2, Supplementary
297 Figure 1C and 1D). For lamb metacarpal, SNP 22142.1 in the same chromosome 16 region had the
298 lowest p value and explained 0.97% of the genetic variance. There was also a single SNP on chromosome
299 3 (OAR3_100483326.1) and a cluster of SNPs on chromosome 19 that had p values smaller than the
300 genome-wide significance threshold and were associated with variation in lamb metacarpal length, with

301 the SNP with the lowest p value from each region explaining 2.08% and 2.40% of the genetic variance
302 respectively (Supplementary Table 2, Supplementary Figure 1E).

303 The two regions on chromosomes 16 and 19 that were associated with lamb metacarpal length variation
304 were also significantly associated with all three leg length measures in adults, with SNP s22142.1 on
305 chromosome 16 and SNP s74894.1 on chromosome 19 respectively explaining 0.80% and 2.04% of the
306 genetic variation in adult foreleg, 0.88% and 1.32% of the genetic variation in adult hindleg, and 0.55%
307 and 2.02% of the genetic variation in adult metacarpal length. There were other regions of the genome
308 also associated with variation in the adult leg length traits; a region on chromosome 11 was significant
309 across all three adult leg length traits, with the most significant SNP explaining 2.35%, 2.25% and 1.13%
310 of the genetic variance in adult foreleg, hindleg and metacarpal respectively (Figure 2B, Supplementary
311 Table 2, Supplementary Figure 1H and 1J). For adult foreleg, a SNP on chromosome 7 and two on
312 chromosome 9 were also associated, with the most significant SNPs in each region explaining 1.31% and
313 2.99% of the genetic variance respectively for this trait (Supplementary Table 2, Supplementary Figure
314 1H).

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

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

319 *Imputed data*

320 Using the SimpleM method (Gao et al. 2008), we calculated the number of effective tests to be 48635,
321 giving a genome-wide significance threshold of 1.03e-06.

322 When performing GWAS using the imputed SNP data, we were able to recover significant SNPs in the
323 same locations for all traits as those we found using the 50K SNP data. Of the 85 SNP-trait associations
324 that we identified with the 50K SNP data, 81 were significant using the imputed SNP data – the
325 remaining four SNPs were no longer significant due to the increased multiple testing burden (which
326 leads to a more stringent significance threshold) between the 50K SNP data and the imputed SNP data
327 ($2.49e^{-06}$ and $1.03e^{-06}$ respectively).

328 We also identified 795 new SNP-trait associations using the imputed SNP data with 425 unique SNPs
329 (Supplementary Table 2). The majority of new associations were in the same regions as the SNPs
330 identified using the 50K SNP data, but we also found new associations: four SNPs on chromosome 1 and
331 three SNP on chromosome 7 was associated with birth weight (Figure 2A, Supplementary Table 2), one
332 SNP on chromosome 3 was associated with adult August weight (Figure 2B, Supplementary Table 2), and
333 one SNP on chromosome 17 was associated with adult metacarpal length (Figure 2C, Supplementary
334 Table 2).

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

336 *Conditional analysis*

337 For any trait that had at least two SNPs on the same region associated with variation in that trait, we
338 fitted the genotype of the SNP with the lowest p value in each region in the GWAS model and removed
339 the SNP from the genotype file. For traits that had multiple SNP associations on more than one

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

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

355

356 *Genes in QTL regions*

357 Given that all of the region-trait associations that were found to be significant with the 50K SNP data
358 were also significant with the imputed SNP data, we chose to focus on top SNPs in the imputed dataset
359 (See Supplementary Table 2 for the list of SNPs, and Supplementary Table 4 for the list of genes).

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

370 We also compared our GWAS results with QTL from Animal QTLdb (Hu et al. 2022). We found that the
371 region on chromosome 6 that we found to be associated with adult August weight overlaps with a
372 region previously found to be associated with carcass weight and final body weight in an (Awassi x
373 Merino) x Merino backcross population (Cavanagh et al. 2010) and is ~0.5Mb upstream of a 2.5Mb
374 region that has also previously been associated with body weight in a population of Australian Merino
375 sheep (Al-Mamun et al. 2015). In addition, the region on chromosome 9 we found to be associated with
376 adult August weight is 1Mb upstream of a region previously found to be associated with live weight in a
377 population of Chinese Merino sheep, however this trait was studied in yearlings rather than adults (Zhao
378 et al. 2021).

379 Chromosome 6 has previously been associated with adult body weight in a smaller population of Soays
380 (Beraldi et al. 2007), however the markers flanking the associated region are not located close to the
381 region we identified.

382

383 *Two-step GWAS*

384 To compare our results using the 50K SNP data to previous GWAS of adult traits in the Soays, we also
385 performed a two-step GWAS. For our two-step analysis, we used the significance threshold previously
386 calculated in Bérénos et al. (2015) ($1.35e^{-6}$).

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

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

397 DISCUSSION

398 Heritability

399 Our results corroborate previous findings that all five body size traits we studied in Soay sheep are
400 influenced by genetic variation in the population (Bérénos et al. 2014), that *post mortem* measures
401 (metacarpal length and jaw length) have higher heritability estimates than live measures (weight,
402 foreleg length and hindleg length), that leg measures have higher heritability than weight (Wilson et al.
403 2006; Beraldi et al. 2007; Bérénos et al. 2014), and that heritability estimates increase with age (Wilson
404 et al. 2006; Bérénos et al. 2014).

405 The heritability estimates for the 50K data were very similar to those estimated using a GRM based on
406 the 50K data in a smaller sample of the same population of sheep by Bérénos et al. (2014), with
407 estimates for the same trait falling within one standard error of each other. The biggest difference was
408 in adult metacarpal length with a heritability difference of 0.05 (estimates were 0.644 (0.047) and 0.594
409 (0.047) for our and Bérénos et al.'s results respectively). Given that we used the same models as
410 Bérénos et al., it is likely that the small differences between heritability estimates for each trait is due to
411 our increased sample sizes.

412 Comparing the heritabilities estimated using the imputed SNP data against the estimates using the 50K
413 SNP data, we found little difference between the two SNP densities in any traits in any age class. The
414 additional genotypes at the imputed SNPs do not give any additional information on additive genetic
415 variation for these traits. This result is not surprising given the previous rarefaction analysis showing that
416 the heritability of these body size traits in adults asymptoted when about half the 50K SNP data was
417 used (Bérénos et al. 2014). There is high LD between nearby SNPs in the Soay sheep genome, which
418 suggests that most, if not all, of the causal variants tagged by the imputed SNP data may have already
419 been tagged by the 50K SNPs. The high LD was reflected when calculating GWAS significance thresholds
420 – whilst the number of SNPs between the 50K SNP data and the imputed SNP data increased by a factor
421 of ten, the number of effective tests only doubled (39K SNPs, 20082 effective tests and 401K SNPs and
422 48635 effective tests respectively).

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

433

434 GWAS

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

437 al. 2021), and several SNPs have already been identified as being associated with variation in these
438 traits. A 2015 study aiming to find SNP-trait associations for these body size traits in adults identified
439 QTL for leg length measures on chromosomes 16 and 19 (s23172.1 and s74894.1 respectively) (Bérénos
440 et al. 2015). A more recent study comparing genomic prediction methods in Soays using the 50K SNP
441 data identified s48811.1 on chromosome 7 and s50107.1 on chromosome 9 as having a probability
442 higher than 0.9 of having a non-zero effect on adult foreleg length in addition to the previously
443 discovered regions on chromosomes 16 and 19 (Ashraf et al. 2021). We were able to identify all four of
444 these associations in our GWAS, alongside associations that have not previously been identified in this
445 population. Use of the imputed SNP data allowed us to discover two more associations with loci that
446 were not genotyped in the 50K SNP data, suggesting that future identification of polymorphisms
447 influencing trait variation in the Soay sheep may benefit from using the imputed data.

448 Performing a two-step analysis confirmed that the novel SNP-trait associations we were able to identify
449 using the 50K SNP data were due to being able to fit the fixed and random effects for each trait whilst
450 performing GWAS all in a single step, rather than the increased population sample. Given the increase in
451 SNP-trait associations when using the single-step methodology, we suggest that a two-step GWAS is
452 redundant with the availability of software like DISSECT which is able to fit fixed and random effects
453 whilst performing GWAS. As we have shown, although DISSECT does not currently have the option to
454 automatically run a repeated measures GWAS, it is possible to modify input files to allow for repeated
455 measures.

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

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

473 We identified 179 genes within 0.5Mb of the top SNPs for each trait (Supplementary Table 4), and of
474 these genes, we found nine that are potential candidate genes for further analyses due to their
475 association with similar traits in other species. However, we stress that it is possible that these nine
476 genes may not be totally responsible for the associations we identified via GWAS – given that we do not
477 have intimate knowledge of genes we identified, we believe that any analyses seeking to confirm gene-
478 trait associations should not just focus on the nine genes listed in Table 2.

479

480 Across all traits for all age classes, the QQ plots showed deviation from the expected distribution of test
481 statistics under the null hypothesis (x=y line) for a wide range of test statistics, including low values,
482 indicative of underlying population structure not accounted for by the GRMs. The first 20 genomic
483 principal components accounted for 10.68% of the variance in the genetic data, and repeating the
484 GWAS analysis fitting these first 20 genomic principal components in addition to the GRM did not
485 change the p values of the SNPs nor the QQ plots. This shows that the principal components in this case
486 were not useful in adjusting for population structure in the presence of the GRM.

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

506 Although we have discovered new SNP-trait associations, it is likely that there are still causative variants
507 that remain undetected. GWAS lacks power to detect rare causative variants and variants with very
508 small effect sizes (Yang et al. 2010). Also, GWAS power drops when the same amount of phenotypic
509 variation is a consequence of multiple variants in the same region as opposed to a single variant
510 (Nagamine et al. 2012). Regional mapping methods have been developed that partition trait variance
511 into regions by simultaneously fitting a whole genome and a regional GRM, with the regions either being
512 defined as fixed SNP windows (Nagamine et al. 2012) or haplotype blocks (Shirali et al. 2018). Such
513 methodologies have the potential to identify regions of the genome that contain variants associated
514 with a trait that are unable to be identified by GWAS either due to being rare, or individually having
515 small effects on trait variation. Genomic prediction, which simultaneously estimates all marker effects
516 drawn from multiple distributions, can also be used to study the genetic architecture of traits by
517 estimating the posterior inclusion probability of a SNP having a non-zero effect on a trait. Genomic
518 prediction has already been used on adult body size traits in Soays, and has identified several of the
519 SNPs we identified through our GWAS approach (Ashraf et al. 2021). Ultimately, we believe that it is
520 important to use a variety of methodologies when studying the genetic architecture of complex traits, as
521 different analyses have different strengths and may be able to identify different QTL.
522

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524

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699

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)	

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

703

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 JMC (Schipani and Provot 2003), EKNS (Duchatelet et al. 2005) and BLC (Schipani and Provot 2003) in humans.
LTF	ENSOARG00000008620	19	Lamb metacarpal length, adult	Human LTF associated with increased bone growth when

			metacarpal length	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).

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

706

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

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

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

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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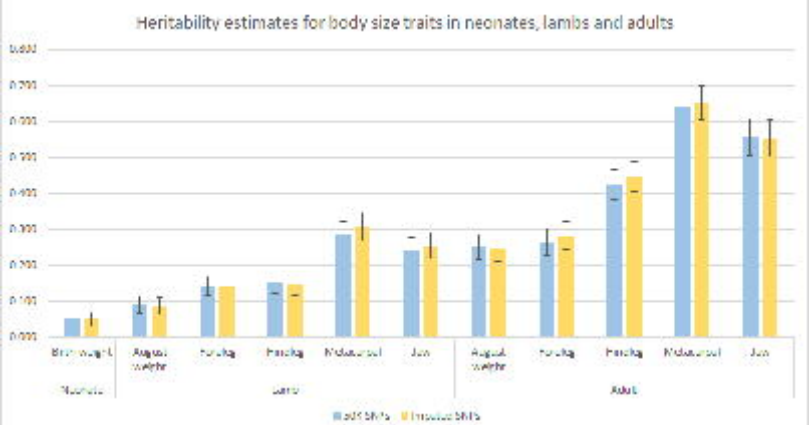
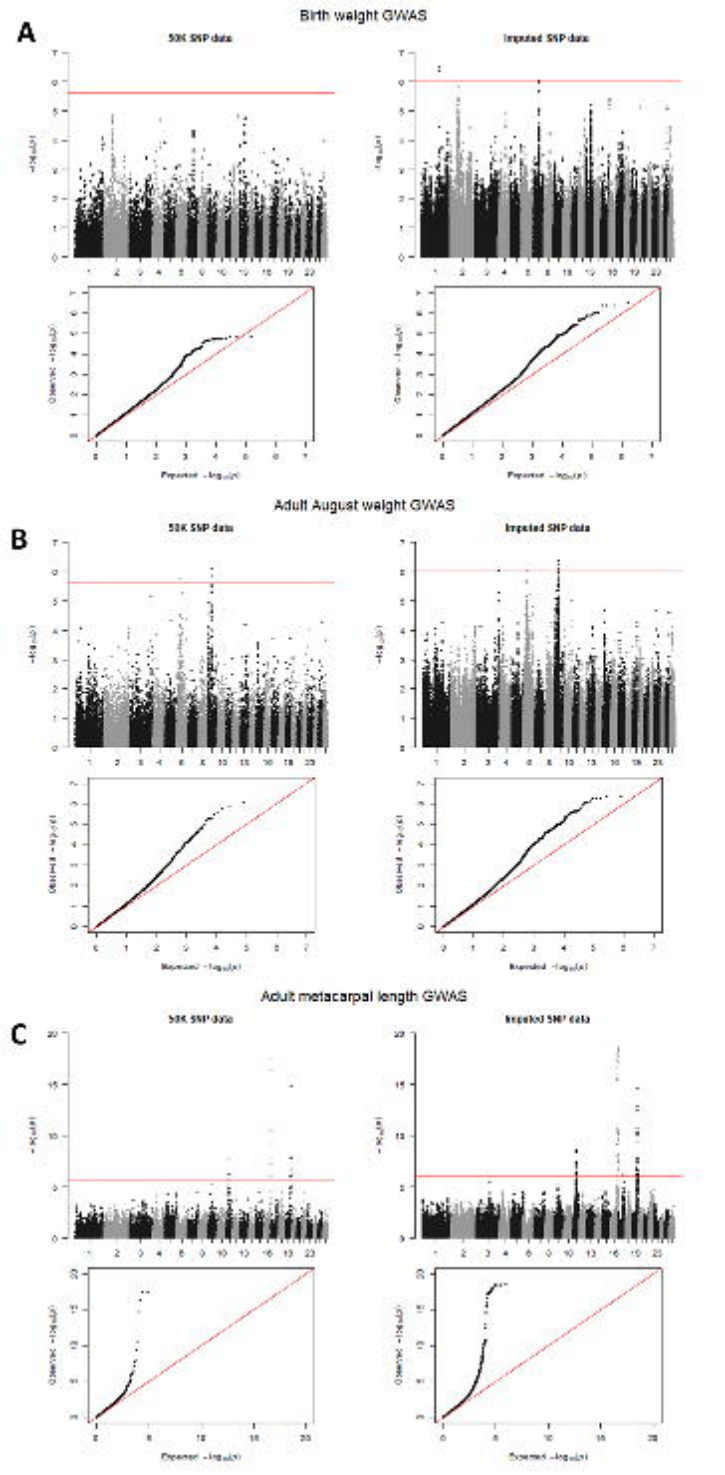
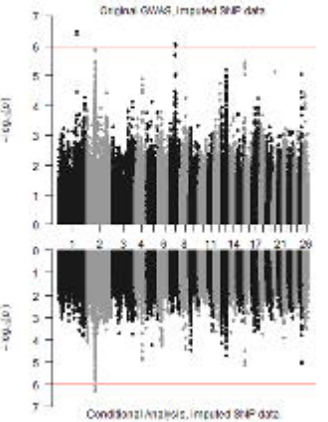
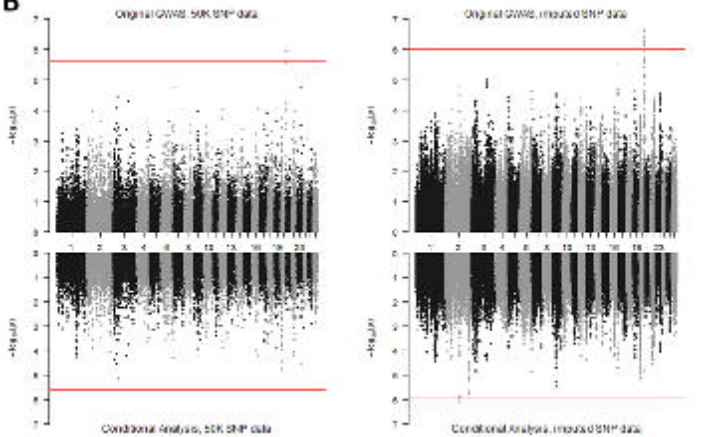

Heritability estimates for body size traits in neonates, lambs and adults


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

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