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1 Variation in mouse pelvic morphology maps to locations enriched in Sox9 Class II and Pitx1
2 regulatory features

3

4 Running Title: Pelvic variation and gene regulation

5

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26

27 **Abstract.** Variation in pelvic morphology has a complex genetic basis and its patterning and
28 specification is governed by conserved developmental pathways. Whether the mechanisms
29 underlying the differentiation and specification of the pelvis also produce the morphological
30 covariation on which natural selection may act is still an open question in evolutionary
31 developmental biology. We use high-resolution Quantitative Trait Locus (QTL) mapping in
32 the F₃₄ generation of an advanced intercross experiment (LG,SM-G₃₄) to characterize the
33 genetic architecture of the mouse pelvis. We test the prediction that genomic features linked to
34 developmental patterning and differentiation of the hind limb and pelvis and the regulation of
35 chondrogenesis are overrepresented in QTL. We find 31 single QTL-trait associations at the
36 genome- or chromosome-wise significance level coalescing to 27 pleiotropic loci. We recover
37 further QTL at a more relaxed significance threshold replicating locations found in a previous
38 experiment in an earlier generation of the same population. QTL were more likely than chance
39 to harbor *Pitx1* and *Sox9* Class II ChIP-seq features active during development of skeletal
40 features. There was weak or no support for the enrichment of seven more categories of
41 developmental features drawn from the literature. Our results suggest genotypic variation is
42 channeled through a subset of developmental processes involved in the generation of
43 phenotypic variation in the pelvis. This finding indicates the evolvability of complex traits may
44 be subject to biases not evident from patterns of covariance among morphological features or
45 developmental patterning when either is considered in isolation.

46
47 **Keywords:** Pelvis; *Sox9*; *Pitx1*; Evolutionary Genetics; Phenotypic Integration; Evolvability.

48 _____
49 Date: February 6, 2019.

50 **Introduction**

51 An outstanding question in evolutionary developmental biology and quantitative genetics
52 is the extent to which development can produce covariation among traits in ways that potentially
53 bias evolutionary trajectories over macro- and micro-evolutionary time scales (Cheverud, 1984;
54 Hallgrímsson, et al. 2009; Rice, 1990; Wolf, 2002). An important step in realizing a unified
55 account of genetics, development, and evolution is building developmentally explicit models of
56 the ways in which developmental processes structure the transformation of genotypic and
57 environmental influences into phenotypic variation (i.e. phenotypic integration) (Cheverud,
58 1984,1996; Hallgrímsson, et al. 2009, 2019; Pigliucci & Preston, 2004; Zeldich, 1988).

59 Comparative morphological and functional analyses of the pelvis have a deep history
60 across multiple fields of study (Grabowski, 2013; Gregory, 1935; Gruss & Schmitt, 2015; Romer
61 & Parsons, 1986). The pelvic girdle forms the anatomical interface between the hind limb and
62 the axial skeleton and serves important roles in bearing the weight of and propelling an organism
63 through its environment. In mammals, the pelvis can show strong sexual dimorphism including
64 in aspects of the morphology of the birth canal, which plays important roles in parturition
65 (Grunstra et al. 2019; McPherson & Chenoweth, 2012). Quantitative genetic investigations into
66 pelvic morphology across several species (Carrier, Chase, & Lark, 2005; Chase et al., 2002,
67 2005; Kenney-Hunt et al., 2008; Kohn & Atchley, 1988) have provided insight into the genetic
68 architecture of variation in this complex skeletal element. Like most aspects of the skeleton,
69 morphology of the pelvis is moderately to highly heritable with robust genetic correlations
70 among traits (Kohn & Atchley, 1988). Likewise, quantitative trait locus (QTL) and other gene
71 mapping analyses show distributions of pleiotropic effects across pelvic traits typical of
72 correlated metric morphological characteristics (Kenney-Hunt et al., 2008; Wagner et al., 2008).
73 While these studies have served to enhance our understanding of the genetic basis of pelvic
74 evolution, the QTL they identify are large and contain many genes, thus yielding limited insight
75 into the location and identity of causative loci and their associated developmental mechanisms.

76 Most recently, these genetic approaches have been supplemented by studies in
77 developmental biology with the aim of identifying the epigenetic processes involved in the
78 specification, differentiation, and growth of the pelvic girdle (Capellini et al., 2011).
79 Developmental investigations into pelvic form have given new insights into the mechanistic
80 basis for the specification and differentiation of the hip bone (i.e. os coxa) and the adjacent,

81 articulated sacrum (Sears, Capellini, & Diogo, 2015; Young, Selleri, & Capellini, 2019).
82 Together, they show three of the pelvis' constituent elements (less the sacrum), a cranially
83 positioned ilium, a caudally/dorsally positioned ischium, and a ventrally located pubis, are
84 specified early in embryonic development via the actions of key transcription factors, including
85 *Pitx1* (Lanctot, Moreau, Chamberland, Tremblay, & Drouin, 1999; Marcil, Dumontier,
86 Chamberland, Camper, & Drouin, 2003), *Pbx1-3* (Capellini et al., 2006, 2011; Selleri et al.,
87 2001), and *Islet1* (Itou et al., 2012), which partition the cells of the early somatopleuric field into
88 distinct cranial and caudal domains. After cell-fate specification, the action of other transcription
89 factors, such as *Sox9* (Bi et al., 2001) and *Emx2* (Malashichev, Borkhvardt, Christ, & Scaal,
90 2005; Malashichev, Christ, & Prols 2008; Pellegrini et al., 2001), in cartilaginous anlagen then
91 lead to cellular differentiation and the onset of endochondral ossification. Numerous signaling
92 molecules interacting between the somatopleure, mesenchymal condensations, and surrounding
93 tissues aid in the development and maturation of the ilium, ischium, and pubis (Young et al.,
94 2019). While these studies provide vitally important insights into the genes and/or regulatory
95 sequences involved in pelvic development, they do not provide an account of how development
96 structures heritable phenotypic variation in pelvic form.

97 Here, we leverage the combined power of high-resolution QTL mapping and functional
98 genomics to investigate the genetic architecture and developmental basis of heritable variation in
99 the mouse pelvis. We integrate quantitative genetic and functional genomic approaches to test
100 hypotheses about the mechanistic basis of the generation of genetic variation on which
101 evolutionary processes might act to effect evolutionary change. Using genotyped and pedigreed
102 individuals from the F₃₄ generation of an advanced intercross design, we first establish the
103 genetic basis of covariation in, and the effects of sex and diet on, eight linear morphological
104 traits reflecting different aspects of ilium, ischium, and pubis morphology (Figure 1). We then
105 identify QTL contributing to individual differences in morphology. Together, these provide a
106 statistical first impression of the genotype-phenotype map for specific parts of the pelvis. Using
107 bioinformatics on developmental genetic and functional genomics features, we next test
108 hypotheses about the relative enrichment of QTL for genes with known roles in the development
109 of the pelvis or bony tissue in general and several classes of regulatory features known to be
110 active in the development of the pelvis and/or hind limb. These tests allow us to generate a
111 refined picture of the phenotypic integration of the pelvis by identifying candidate mechanisms

112 for the conversion of genomic variation into phenotypic variation in pelvic morphology.

113

114 **Materials and methods**

115 *Animal subjects and care.* All experiments using mice were approved by and conducted in
116 accordance with the standards of the Institutional Animal Care and Use Committee (IACUC) of
117 Washington University School of Medicine, St. Louis. Mice used in this study were acquired
118 from the F₃₄ generation of an advanced intercross (AI) experiment (LG,SM-G34) descended
119 from an initial cross of LG/J females and SM/J males obtained from The Jackson Laboratory. F₁
120 hybrids from this cross were then intercrossed to produce an F₂ generation. From the F₂
121 generation onwards, the animals were mated at random except to avoid brother sister pairs and
122 minimize variation in the contribution of full sibships to the next generation. Half of the
123 individuals in each sex in each litter were fed a high fat (#TD88137, Harlan Teklad) or low fat
124 diet (#D12284, Research Diets) with similar caloric content starting at weaning (at 3 weeks of
125 age). Detailed explanations of the breeding and handling of the mice can be found in Norgard et
126 al. (2011). The F₃₄ generation includes \approx 990 skeletonized individuals from 137 full sibships
127 depending on the measured characteristic (see Table 1 for sample size and summary statistic
128 information). Carcasses were skinned and skeletonized using dermestid beetles.

129

130 *Genotyping.* Each individual mouse was genotyped for 2,842 single nucleotide polymorphisms
131 distributed across the autosomal genome. These SNPs were drawn from the Oxford/CTC SNP
132 set (<http://www.well.ox.ac.uk/mouse/INBREDS/>) and are all polymorphic between LG/J and
133 SM/J inbred mouse strains. The allelic states of SNPs were assessed using the Illumina
134 GoldenGate Bead Array (Illumina, San Diego, CA) at the Center for Inherited Disease Research.
135 The SNPs were spaced at intervals averaging \approx 0.5 centiMorgans (cM) scaled to the F₂ map and
136 \approx 8.5cM scaled to the F₃₄ map.

137

138 *Phenotyping.* Individual hip bones (ossa coxae) were embedded in florist foam forms and
139 subjected to micro-CT scanning at 34.5 μ resolution using a Skyscan 1172 (Bruker) μ CT. The
140 images were reconstructed in NRecon (Bruker) and then processed, visualized, and scored for
141 three-dimensional coordinate data at landmarks using AMIRA (ThermoScientific). A set of eight
142 linear distances among landmarks was used in this analysis. These were chosen to cover

143 dimensions of the pelvic bone to reflect overall form and function of the pelvis, to capture
 144 dimensions related to the structure of each pelvic sub-element (i.e., ilium, ischium, and pubis),
 145 and to capture traits figuring prominently in arguments about the evolution of the pelvis in
 146 vertebrates, especially humans and other mammals (Figure 1). All measurements on all
 147 individuals were taken by a single individual. In the case of several traits, multiple inter-
 148 landmark distances were averaged to minimize the effect of intra-observer measurement error.
 149 These include the lengths of the ischium, ischium to caudal iliac blade, pubis length, ischium to
 150 pubis length, and pubis to cranial ilium (see Figure 1 for details). A subset ($n = 72$) of the bones
 151 were scanned, reconstructed, and measured twice to assess the repeatability of the results.
 152 Repeatabilities in the form of within individual cross-replicate variance were estimated for each
 153 trait using ANOVA (Sokal & Roff, 1995). Individual traits were highly repeatable, nearly always
 154 exceeding 95%, and the averaged traits showed even higher repeatabilities.

155
 156 *Quantitative genetic analysis.* Additive genetic variance-covariance matrices were estimated
 157 using a mixed effects model fit with the MCMCglmm package (Hadfield, 2010) in the R
 158 statistical computing environment (R Core Team, 2018). An additive genetic relationship matrix
 159 among individuals obtained using a pedigree was used to model the random effect of relatedness.
 160 Covariates consisted of terms for diet (either high- or low-fat), sex (male or female), whether an
 161 individual came from a large or small litter (a number of pups equal to or fewer than vs. greater
 162 than the number of nipples on a dam), and age at sacrifice (in days). We used a weakly
 163 informative prior with a degree of belief parameter equal to the number of traits assuming a
 164 heritability of 0.5 for all traits and no covariance among them.

165 Posterior distributions for the model parameters were obtained in each case by sampling
 166 over 1,000,000 iterations using a thinning interval of 500 after a burn-in time of 500,000
 167 iterations. We used the posterior distributions to calculate estimates of narrow sense heritability
 168 (h^2), evolvability (e , Hansen & Houle, 2008), and the additive genetic (**G**) and environmental (**E**)
 169 covariance. Convergence was assessed by inspecting the plots of the traces of the Markov chain
 170 and ensuring auto-correlation across samples was acceptably low for all terms ($r \approx 0.1$).

171
 172 *QTL mapping.* We estimated the locations of QTL using a mixed model extension of the Haley-
 173 Knott (Haley & Knott 1992) method, tailored for use in advanced intercross experiments using

174 the QTLRel package (Cheng, 2011). Probabilities of genotypic scores were interpolated between
175 scored markers at 1 cM (on the F₃₄ scale. \approx 0.06 cM on the F₂ scale) intervals between loci
176 containing scored SNPs. We fit models at each marker location and each imputed intervening
177 cM location. The covariates age, litter composition, sex, and diet were included in the model.
178 Additive (*a*) and dominance (*d*) genetic effects and their standard errors were estimated using a
179 generalized linear model controlling for covariates and relatedness among individuals. Unlike in
180 the F₂ generation of an intercross experiment, later generations have family structure. As such,
181 we include the combined polygenic effect of all genes on individual differences in the model. We
182 did so by using additive and dominance relationship matrices drawn from the same pedigree used
183 to estimate the genetic covariance among the traits.

184 We fit genetic models including additive and dominance effects on each trait at each
185 genotyped locus and imputed intermediate position. We compared the fit of the basic model for
186 each trait at each imputed location in the genome to the fit of a null model including no genetic
187 terms using a likelihood ratio test and expressed the differences in fit as a LOD score.

188 Thresholds for acceptance of a region as a QTL were estimated by adjusting the
189 minimum acceptable LOD value using a Bonferroni correction based on the effective number of
190 loci on each chromosome (Li & Ji 2005). The seventeen-fold increase in the length of the genetic
191 map of the F₃₄ generation over the map of the F₂ generation of the same population means some
192 individual chromosomes in the F₃₄ are about as long as the entire F₂ genome. As such, we
193 followed the suggestion of Chen and Storey (2006) and chose the chromosome-wise threshold to
194 accept QTL. The chromosome-wise thresholds ranged from 3.66 on chromosome 1 to 2.92 on
195 chromosome 19 and the genome-wise threshold was 4.72 (Table 2). We used a relaxed threshold
196 to evaluate the LOD scores within regions identified in a study of pelvic traits in the F₂
197 generation of the same population (Kenney-Hunt et al., 2008). Given the subtle differences in the
198 traits and the ways in which they were measured, we set the relaxed threshold to LOD = 2.8,
199 reflecting eight chances from eight traits to find an effect within the bounds of a single QTL
200 discovered in the F₂. This allows evaluation of the replication of results across generations and
201 provides an expanded set of regions to test for enrichment of genomic properties (see below). We
202 used a 1.5 LOD drop-off criterion to identify the confidence regions for QTL (Manichaikul et al.
203 2006).

204 The additive and dominance effects of QTL on each trait and their standard errors were

205 estimated using a generalized linear model. In cases where there were pleiotropic effects of a
206 QTL across multiple traits, each with a slightly different peak location within the QTL, the
207 effects on the respective traits were estimated using the location corresponding to their individual
208 highest LOD score. The genomic location of each QTL interval was expressed in terms of mouse
209 genome version mm9.

210

211 *Functional genomics analysis.* Each QTL was queried against the MGI Gene Expression Data
212 resource (Smith et al., 2014) to identify those genes: a) Expressed in the developing pelvic
213 girdle; b) Expressed in skeletal tissues in general; and c) In the MGI Mutant Allele Function
214 resource (Eppig et al. 2015) of mutants affecting the pelvis or the growth of bone. We also drew
215 on published gene enhancer locations (see below) known to be associated with limb formation
216 and another set of the same verified in VISTA (Infante, 2015). We recorded which elements lay
217 within the bounds of each of the QTL by category to achieve a qualitative sense of the overlap
218 between genes suspected to play central roles in pelvic/bone development and those regions of
219 the genome we identify as contributing to individual differences in pelvic morphology.

220 In a second analysis, we tested to see if the QTL identified were enriched for these
221 features relative to random locations in the genome. We examined potential Pbx1 and Emx2
222 dimerization motifs (Capellini, et al. 2011), ChIP-seq identified Pitx1 binding peaks (Infante,
223 Park, Mihala, Kingsley, & Menke, 2013), ChIP-seq identified Sox9 binding peaks (Generic and
224 Class I and II. Ohba, He, Hojo, & McMahon, 2015; Liu et al., 2015), Sox9 SuperEnhancers (Liu
225 & Lefebvre, 2015; Ohba et al., 2015), H3K27ac marked regulatory elements (of the flank and
226 hind limb expressed at age E11.5. Infante et al., 2013), and DNase I hypersensitivity data from
227 the ENCODE database generated on hind limb and flank tissues (ascertained at age E11.5.
228 ENCODE Consortium et al., 2007).

229 These different sets of genomic features were systematically assembled and examined for
230 intersections with QTL using the UCSC Table Browser Intersection Tool (Karolchick, 2004), as
231 opposed to being collected from the literature and is thus not subject to biases inherent to
232 happenstance collection. We used a χ^2 test to see if each class of elements was represented in the
233 QTL more often than we would expect if drawn from the entire genome by chance. We
234 conducted separate tests on the results derived from the F₃₄-only results and the results including
235 replicated results across the F₂ and the F₃₄. We performed a total of 13 tests for each set of QTL,

236 one for each of the class of features suspected to be involved in the development of the pelvic
237 girdle, bone development, and/or hind limb and flank development, and one for the occurrence
238 of known or suspected genes based on functional effect or expression. We judge significance
239 using a Bonferonni-adjusted significance threshold for 13 tests ($\alpha \approx 0.0038$).

240

241 *Data availability statement* The phenotypes, covariates, pedigree, and genotypes used in this
242 study will be made available on the Dryad repository in the event that the paper is accepted.
243 These data may also be obtained on request to the corresponding author.

244 **Results**

245 As is the case with most morphological features, we find there is ample genetic variation in
246 pelvic traits underlain by many loci, each with a small effect. The QTL identified in the F₃₄
247 strongly replicate those found in an earlier generation (F₂) of the same experimental population.
248 We find few genes with known or suspected roles in development of pelvis/hind limb in the
249 QTL. The QTL are, however, enriched in some classes of regulatory features known to be active
250 in pelvis and hind limb development.

251

252 *Ample genetic variation in the pelvis.* Summary statistics for the linear pelvic traits in the
253 population are presented in Table 1. All traits were moderately to highly heritable and had
254 evolvabilities well within the range of values observed for morphological traits (Table 1),
255 especially those reflecting skeletal form (Cheverud, 1988; Hansen, Pélabon, & Houle, 2011).
256 The evolvability estimate for Ilium Width stood out as being considerably larger than those
257 calculated for other traits. Otherwise, the relative values of the estimates of the magnitude of
258 additive genetic variation were not particularly different among pelvic traits. The genetic and
259 phenotypic correlations showed strong similarities in their distribution among traits (Matrix
260 correlation of $R = 0.92$ with a posterior credible interval of 0.86 to 0.96) as is typical for
261 morphological traits when sample sizes are large (Cheverud, 1988).

262

263 *Multiple loci of small effect contribute to genetic variation in the pelvis.* For the analysis using
264 only results from the F₃₄ generation, we detected 31 single trait QTL significant at the genome-
265 or chromosome-wise significance threshold across 12 of the 19 autosomes, which coalesce into
266 27 pleiotropic QTL (see Table 3 for a summary of their locations and their effects). Six QTL

267 reached genome-wide significance ($\text{LOD} \geq 4.7$). The QTL spanned regions ranging between 1.1
 268 and 10.0 Mb. Most of the QTL affected only single traits at this level with two having significant
 269 effects on two traits (QTL *Pelvis* 4.4 affecting Ischium to Caudal Iliac Blade and Pubis to Ilium
 270 Length; *Pelvis* 12.2 affecting Ilium Length and Ischium Length) and one affecting three traits
 271 (QTL *Pelvis* 13.2 affecting the traits Ischium to Caudal Iliac Blade, Ischium Length, and Ischium
 272 to Pubis Length). Most loci, however, had significant effects on other traits at a pointwise ($p =$
 273 0.05) level in detected QTL, indicating some degree of pleiotropy. These results are similar to
 274 those obtained by previous studies of skeletal traits, which resulted in a small number of highly
 275 pleiotropic QTL (Wagner et al., 2008).

276 In general, QTL had statistically significant additive effects with the Lg/J derived allele
 277 tending to increase the value of the trait. Of the 31 single trait QTL in the F_{34} generation, 27 had
 278 statistically significant additive effects, 21 of which indicated the allele derived from the Lg/J
 279 strain increased the value of the trait relative to the Sm/J derived allele. The magnitude of the
 280 additive effects averaged 1.1% of the mean of their respective traits, ranging from 0.2% to 3.9%.
 281 Dominance effects were also evident, with 12 of 31 single trait QTL in the F_{34} exhibiting
 282 statistically significant dominance effects and three displaying signs of under- or over-
 283 dominance. Dominance effects averaged 0.75% of the mean and ranged from 0% to 3.5%. On
 284 average, each single QTL accounted for 3.1% of the phenotypic variance of its respective trait,
 285 with a minimum of 1.9% and maximum of 12.3%.

286
 287 *Strong replication of results across generations.* Comparing the results of the present study to a
 288 similar study conducted on the F_2 generation of the same population (Kenney-Hunt et al., 2008)
 289 yielded substantial overlap between the two studies. Of 27 QTL (discounted for pleiotropy)
 290 identified in this study, 18 of them replicated results from the F_2 generation of the same
 291 population (Table 3). Moving from the F_2 generation to the F_{34} generation, 42 of 58 of the
 292 Kenney-Hunt (Kenney-Hunt et al., 2008) QTL replicated for at least one pelvic measurement in
 293 the F_{34} with a $\text{LOD} \geq 2.3$ (Supplementary Table 1). However, a conspicuous mismatch between
 294 the two studies is apparent in the results on chromosome 12, where it appears that several QTL
 295 with opposite additive effects not apparent in the F_2 now emerge in the F_{34} generation.
 296 Recombination in the intervening generations might have led to them segregating with sufficient
 297 independence to become distinguishable in the F_{34} generation.

298

299 *Few genes with known roles in bone development reside in QTL.* In the QTL identified in the F₃₄
300 analysis and the QTL from the combined F₂/F₃₄ analysis, we found there was a modest
301 representation of genes with known roles in pelvic or bone development, as assessed by
302 intersections with databases on gene expression and function (Table 4 and Supplementary Table
303 2). Interestingly, many of the genes implicated as having core roles in the early patterning of the
304 pelvic girdle, which include genes known to affect the patterning of the pelvis (Young et al.,
305 2019) including the ilium (*Tbx4*, *Emx2*, *Fgf10*, and *Pbx1/2*), the pubis (*Alx1/4*, *Prrx1*, and
306 *Twist1*), and possibly the ischium (*Pax1*) were absent from QTL. However, residing within these
307 intervals are two factors playing notable roles throughout pelvic development particularly at
308 mesenchymal condensation and chondrogenesis stages. The first is *Sox9*, present on
309 chromosome 11 in a QTL influencing Ischium Length (QTL *Pelvis 11.3*), which reaches
310 chromosome-wise significance (LOD = 3.56). The second is *Pitx1*, present on chromosome 13 at
311 335 cM, well within the bounds of the QTL influencing Ilium Width detected using the
312 combined F₂/F₃₄ analysis (*Pelvis F2/F34 13.01*, Supplementary Table 2).

313

314 *QTL are enriched with regulatory features involved in aspects of pelvic development.* We tested
315 whether our identified QTLs are enriched for genomic features known to be involved in the
316 development of pelvic, limb, and bone development. To carry out these analyses we first mined
317 several published transcription factor and histone marker ChIP-seq, DNase I HS, and *in silico*
318 transcription factor prediction datasets on developmental regulation of limb development (see
319 methods) and matched their locations with QTL intervals and the genome-wise distribution of
320 elements. Judged against a Bonferroni-corrected significance level ($p = 0.0038$), five of twelve
321 classes of features known or predicted to play a role in the regulation of pelvic girdle, skeletal,
322 and limb development were more frequent in the QTL identified in the F₃₄ alone than was
323 expected by chance (Table 5). In addition, six of twelve classes were overrepresented in QTL
324 from the combined F₂/F₃₄ analysis (Table 5). Specifically, we found *Pitx1* ChIP-seq signals,
325 reflective of the locations in the genome where *Pitx1* physically binds during hind limb
326 development, were particularly strongly enriched within the QTL. Likewise, *Sox9* Class II ChIP-
327 seq peaks, indicative of *Sox9* binding in chondrocytes, were also highly enriched relative to
328 chance in both tests. In the results from the F₃₄ alone, generic *Sox9* ChIP-seq peaks, as identified

329 by Liu et al. (2015), were also enriched at the Bonferroni-corrected level but only at the single
 330 test ($p = 0.05$) level in the combined F_2/F_{34} QTL. Conversely the Sox9 Class I ChIP-seq peaks
 331 were not overrepresented in QTL from the F_{34} only analysis but were in the combined F_2/F_{34}
 332 QTL. With respect to histone ChIP-seq assays on the E11.5 limb and flank using H3K27ac,
 333 typically considered a marker of active enhancers, only those called hind limb peaks were
 334 enriched and only in the combined F_2/F_{34} QTL analysis. Despite their hierarchical roles in pelvic
 335 patterning, *in silico* predicted Pbx/Emx2 binding sites were not enriched in either the F_{34} QTL
 336 alone or in the combined F_2/F_{34} results when multiple tests were taken into consideration.
 337 Finally, known genes were overrepresented in QTL in both cases.

338

339 **Discussion**

340 The principle innovation of this study lies in the connection between variation (QTL) and
 341 developmental genetic (i.e., regulatory mechanisms) accounts of pelvic morphology. Our first
 342 goal was to characterize the genetic basis of variation in the mouse pelvis using statistical
 343 quantitative genetic and QTL mapping techniques. A second goal was to assess the extent to
 344 which a QTL-based quantitative genetic account of the generation of individual differences
 345 showed signs of being structured by different classes of developmental processes. This was
 346 accomplished by way of testing to see if the QTL identified here contained genes from a suite
 347 known to be involved in pelvic or limb development. Likewise, we tested to see if the QTL
 348 identified here were enriched for a series of features that were drawn from the developmental
 349 literature and suspected to be involved in pelvic development.

350 We found variation in the mouse pelvis is a complex interplay of many genetic and
 351 environmental influences acting through the life course, as has been the case for other
 352 morphological characteristics (Cheverud, 1988; Kruuk, Slate, & Wilson, 2008). Moreover, not
 353 all developmental pathways involved in the development of the pelvic girdle appear to channel
 354 the genetic influence responsible for generating individual differences in this population. This
 355 conclusion, however, is tempered by our relative dearth of knowledge on the developmental
 356 genetic mechanisms governing the pelvis, as compared to the limb for example (Sears et al.,
 357 2015; Young et al., 2019). Thus, knock-out and other experiments using pelvic gene mutations of
 358 large effect may not afford clear insight into the mechanisms that can vary in natural populations.

359 It is important to emphasize that our results depend on the particular QTL we identify. If

360 animals are drawn from different original stocks, they will likely display different patterns of
361 genetic variation. As such, other experiments might identify different regions of the genome as
362 being important for pelvic variation and these regions might contain different genomic elements
363 than those identified here. Whether these alternate suites of QTL might display rather different
364 patterns of association with genomic elements is an empirical issue resolvable by replicating this
365 study in another experimental population.

366

367 *Variation in pelvic morphology is caused by many loci of small effect.* With respect to the
368 additive genetic covariation, the morphology of the pelvis is highly to moderately evolvable and
369 heritable and shows substantial genetic correlations among traits. As is typical for metric
370 morphological traits, the QTL tend to be detected for a single trait with chromosome- or genome-
371 wise significant effects (Wagner et al., 2008) with few highly pleiotropic loci. Each QTL also
372 accounted for a small proportion of the total genetic variance in each trait with no one trait's
373 variance being fully accounted for by the effects of QTL.

374 The additive effects of QTL show the substitution of an allele derived from the large
375 founder strain for one from the small strain tended to increase the value of the trait, which is
376 consistent with the evolved differences between the founding strains. There are, however, a fair
377 number of exceptions in which the SM/J derived allele imparts an increase on the trait value. The
378 change in genomic background and developmental context brought on by the crossing of the two
379 strains might have led to novel epistatic interactions and changes in the effects of the alleles.
380 Alternatively, alleles with effects contrary to the direction of selection in either founder strain
381 may have been fixed by random genetic drift or drafted along with linked alleles of stronger
382 effect during the selection used to produce the strains.

383

384 *Skeletal growth rather than patterning may generate evolvability of pelvic morphology.* To date,
385 most of what is known about the developmental genetics of the pelvis relates to the roles of early
386 transcription factors and signaling molecules during the bone's patterning stage. Indeed, this
387 stage has been most intensively studied because of the finding that the pelvic field is closely
388 affiliated with the early limb field (Capellini et al., 2011; Sears et al., 2015). Thus, it is not
389 surprising that our understanding of the patterning mechanisms of pelvic development has been
390 influenced by targeted studies in limb development, which have characterized factors necessary

391 for the development of both structures.

392 Conspicuously absent from our pelvic QTL are many genes with known involvement in
393 the basic patterning of the pelvic girdle even though there are several transcription factors with
394 specific roles in the development of the individual pelvic elements. This may arise from a partial
395 separation between the action of gene regulatory networks and other developmental pathways
396 responsible for laying out the basic patterning of a developing structure and those influencing
397 variation through growth among non-pathological adults. Of a set of genes identified as playing a
398 crucial role in the patterning of the mouse pelvic girdle by Young et al., (2019), Sears et al.
399 (2015), and others (Capellini et al., 2006, 2011; Itou et al., 2012; Lanctot et al., 1999; Marcil et
400 al., 2003; Selleri et al., 2001), including *Emx2*, *Fgf10*, *Pbx1/2*, *Pitx1*, *Sox9*, and *Tbx4* in ilium
401 patterning, *Alx1/3/4*, *Islet1*, *Prrx1*, and *Twist1* in pubis patterning, *Pax1* and *Islet1* in ischium
402 patterning, only *Sox9* was included in a QTL identified in the F₃₄-only analysis while *Pitx1* was
403 found using additional results from the F₂. These results are contingent, in part, on the particulars
404 of the population in which we mapped the QTL. Different experiments on crosses of other mouse
405 strains might well lead to different QTL being discovered and different sets of genes might be
406 represented in those QTL.

407 The lack of known genes with central roles in patterning marking the early development
408 of the pelvis may indicate the patterning stages are not periods of development during which
409 proper function of the organism can tolerate variation and thus constitute constraints on
410 evolution via internal stabilizing selection (Cheverud, 1984). This difference in variability (i.e.
411 the propensity for a system to generate variation) between early processes of patterning and later
412 processes including endochondral ossification is supported at the genomic level in part by a lack
413 of enrichment in many genomic elements related to the patterning stage of limb and pelvis
414 development. We interpret this result as indicating that variation in the skeletal morphology of
415 the mouse ilium, ischium, and pubis may not be generated during early stages of pelvic
416 patterning but rather at later stages involved in endochondral bone growth and ossification (See
417 also Sanger et al., 2011). Future work targeting both developmental windows for the locations of
418 important transcriptomic and epigenomic signatures along with functional and biomechanical
419 analysis will address this issue more concretely.

420

421 *Comparing variational and functional genomic results.* Little is known about the developmental

422 processes that permit the generation of variation in the pelvis. The results of our investigation
423 into the developmental correlates of variation demonstrate that not all classes of genomic
424 features known to play some role in the development of the pelvis and/or limb appear to be
425 enriched in the QTL we identify as contributing to variation in the pelvis in this population. As
426 elaborated on above, we emphasize “in this population” as the particular associations between
427 genes and other genomic features and regions of the genome identified in QTL analyses may
428 differ across experiments.

429 In the case of *Pbx/Emx2* sites, where we do not find significant enrichment of the QTL
430 after considering multiple tests, Capellini et al. (2011) reported that mutations to the different
431 *Pbx* genes often resulted in complex pelvic phenotypes (i.e., those influencing both cranial and
432 caudal pelvic structures, and more often than not resulting in complete loss of pelvis rudiments
433 during development), not simply by additively regulating variation in pelvic girdle morphology.
434 In combination with our results, this would suggest that while *Pbx* family members
435 hierarchically regulate various downstream factors, including *Emx2*, and factors responsible for
436 ilium and pubis patterning (Capellini et al., 2011), phenotypic variation arises through other
437 inputs into these systems. In this scenario, genetic influences resulting in variation in adult
438 phenotypes may not be introduced into the population through this set of mechanisms, perhaps
439 because of this (i.e., *Pbx/Emx2*) network’s overarching effects on both cranial and caudal pelvic
440 structures. On the other hand, influences acting through *Sox9* and potentially its target genes
441 during chondrogenesis appear to be important for the generation of genetic variation in this
442 population. We also found that E11.5 HL/Flank H3K27ac signals, which serve to mark active
443 enhancers, were also not overrepresented in our QTL. This may indicate that, while this kind of
444 epigenetic modification may be important for the specification of the hind limb and pelvic girdle,
445 it may not serve as a mechanism by which genetic variation in pelvic form is generated.

446 While *Sox9* SuperEnhancers do not appear to be overrepresented in our QTL and we get
447 mixed results for generic *Sox9* ChIP-seq results (Liu et al., 2015), we have a clear signal for the
448 enrichment of *Sox9* Class II ChIP-seq peaks (Ohba et al., 2015) in our QTL in both the F₃₄ only
449 analysis and in the combined F₂/F₃₄ analysis. The *Sox9* Class II features are known to be highly
450 tissue specific and involved in regulating chondrocytes through the direct binding of *Sox9*
451 complexes to the DNA itself (Pellegrini et al., 2001), thus making the *Sox9* Class II regulatory
452 mode a prime candidate mechanism for understanding how individual differences in pelvic form

453 are generated. In this scenario, allelic variation that modifies Sox9 binding events may be
454 governing pelvic variation. As Sox9 is a key regulator of chondrogenesis, these results also
455 support the hypothesis that endochondral ossification and the growth it promotes may more
456 likely be targeted by evolutionary processes than early patterning.

457 The *Pitx1* gene has an important role in specifying the hind limb or pelvic fin structures
458 across vertebrates and has been implicated as a primary contributor to variation in the pelvic
459 apparatus in populations of stickleback fish enabling them to adapt quickly to different
460 environments (Chan et al. 2005; Thompson et al., 2018). Moreover, mutations to *Pitx1* in mice
461 result in ilium dysmorphologies among other more subtle pelvic and hind limb alterations
462 (Marcil et al. 2003). The weight of evidence from developmental investigations in mammals
463 suggest *Pitx1* plays multiple roles in regulating the endochondral bone growth in the hind limb
464 through both the proliferation of chondrocytes and their terminal differentiation thus laying down
465 the conditions for ossification. This makes it a prime candidate for a process important for
466 driving the generation of phenotypic variation (Infante et al. 2013; Marcil et al. 2003).

467 Our results support this contention as all classes of Pitx1 ChIP-seq features were
468 overrepresented in the QTL identified here indicating regulation of growth through this set of
469 mechanisms may contribute to individual differences in this population of mammals. In the case
470 of *Pitx1* and the evolution of the pelvic apparatus in stickleback fish, the *Pitx1* gene itself shows
471 signs of being highly conserved, with adaptive differences among groups being driven by natural
472 selection acting on variation in its associated regulatory features (Chan et al., 2005; Thompson et
473 al., 2018). Our results support the position that *Pitx1* *cis*- and *trans*-regulation is an important
474 regulator of phenotypic integration in the pelvic girdle and may be important for rendering the
475 pelvic girdle evolvable across many species of vertebrates. This latter point is supported by our
476 finding of a QTL affecting Ilium Width in the F₂/F₃₄ replicated set containing the *Pitx1* gene
477 (QTL *Pelvis F₂/F₃₄ 13.01*). Ilium Width is the most evolvable structure in our dataset and the
478 only trait standing out from the rest in terms of its variational properties.

479

480 **Conclusion**

481

482 Mechanisms underlying the basic patterning of morphology may not always be involved in the
483 generation of covariation among traits available for natural selection and random genetic drift to

484 cause evolutionary change. Here, we demonstrated Pitx1 and Sox9 Class II regulation are
485 important mechanisms for the phenotypic integration of the mouse pelvis. That Pitx1 regulation
486 may be an important developmental mechanism for the generation of covariation in this mouse
487 population is particularly exciting given its role in structuring variation allowing adaptation of
488 the pelvic apparatus of populations of stickleback fish to new environments (Chan et al., 2005;
489 Thompson et al., 2018).

490 While the genetic basis of covariation in the mouse pelvis is complex and the product of
491 many overlapping influences acting through development, developmental mechanism-specific
492 channeling of genetic influences on morphology may lead to strong biases in the ways in which
493 covariation can occur in populations given different distributions of segregating alleles and
494 interactions with the environment (Cheverud, 1984). These biases in the generation of
495 covariation through development might have both constrained and enabled the evolutionary
496 trajectories leading to the diversity of pelvic girdle morphology we see today.

497

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