Complexities of recapitulating polygenic effects in natural populations: replication of genetic effects on wing shape in artificially selected and wild caught populations of *Drosophila melanogaster.*

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Abstract

Identifying the genetic architecture of complex traits is important to many geneticists, including those interested in human disease, plant and animal breeding, and evolutionary genetics. Advances in sequencing technology and statistical methods for genome-wide association studies (GWAS) have allowed for the identification of more variants with smaller effect sizes, however, many of these identified polymorphisms fail to be replicated in subsequent studies. In addition to sampling variation, this failure to replicate reflects the complexities introduced by factors including environmental variation, genetic background, and differences in allele frequencies among populations. Using *Drosophila melanogaster* wing shape, we ask if we can replicate allelic effects of polymorphisms first identified in a GWAS (Pitchers et al. 2019) in three genes: *dachsous (ds)*, *extra-macrochaete (emc)* and *neuralized (neur),* using artificial selection in the lab, and bulk segregant mapping in natural populations. We demonstrate that multivariate wing shape changes associated with these genes are aligned with major axes of phenotypic and genetic variation in natural populations. Following seven generations of artificial selection along the *ds* shape change vector, we observe genetic differentiation of variants in *ds* and genomic regions containing other genes in the hippo signaling pathway. This suggests a shared direction of effects within a developmental network. We also performed artificial selection with the *emc* shape change vector, which is not a part of the hippo signaling network, but showed a largely shared direction of effects. The response to selection along the *emc* vector was similar to that of *ds*, suggesting that the available genetic diversity of a population, summarized by the genetic (co)variance matrix (**G**), influenced alleles captured by selection. Despite the success with artificial selection, bulk segregant analysis using natural populations did not detect these same variants, likely due to the contribution of environmental variation and low minor allele frequencies, coupled with small effect sizes of the contributing variants.

Introduction

Dissecting the genetic architecture underlying complex traits remains challenging, because of the joint contributions of many alleles of small effect, genotype-by-environment interactions, and other factors. Progress in sequencing technology in conjunction with development of GWAS statistical methodologies has enabled identification of loci contributing to numerous complex traits and diseases. However, such mapping approaches identify only a subset of loci contributing to trait variation (Visscher et al., 2017). In part, this reflects the low power to detect rare alleles, and those with small effects (Tam et al., 2019). For alleles that are relatively common in a population, replication rates between GWAS studies are high, even when effect sizes are small (Marigorta et al., 2018). However, GWAS studies have failed to replicate the effects observed in many candidate gene studies, in part due to the fact that many alleles identified in these studies are rare in populations, and require very large cohorts to detect (Fritsche et al., 2016; Ioannidis et al., 2011).

In cases where an association is replicated between studies, the magnitude of the effect can vary substantially between different cohorts or populations (CONVERGE consortium, 2015; Marigorta et al., 2018). Differences can arise because of genetic background due to epistatic gene by gene (GxG) interactions, or due to gene-by-environment (GxE) interactions. The initial estimates of effect size will be biased upwards if statistical testing in the initial cohort is used to determine which SNPs are chosen for replication studies. It is important to understand which of these causes of differences in effect size are of practical significance when we want to generalize results to different populations or environments.

In this study, we focus on the issue of replication in a multivariate context, where the joint inheritance of multiple features are simultaneously investigated. We will refer to the suite of measured features as a 'multivariate trait' for convenience. In this case, what we want to estimate is the vector of effects of each SNP on all measured features. Each SNP may have a unique combination of effects. Univariate effects vary only in magnitude, as we can only infer effects on a single feature. For a multivariate trait, estimated genetic effects vary in magnitude, the sum of effects on all traits, and also in direction, how the total effect is allocated among different features (Melo et al., 2019). The ability to study the direction along with the

magnitude of genetic effects provides an additional and important way of assessing repeatability. For a univariate trait, there is a 50% chance that the replicate estimate will be in the same direction as the original estimate, even with no true effect. By contrast, the probability of a "replicated" genetic effect sharing a similar direction by chance alone decreases as the number of measured features increases (Marquez and Houle, 2015; Stephens, 2013).

Studying genetic effects in a multivariate context is beneficial in other ways. First, it has been demonstrated both empirically and via simulations, that genetic mapping for multivariate traits generally increases statistical power over trait by trait analyses (Fatumo et al., 2019; Pitchers et al., 2019; Porter and O'Reilly, 2017; Shriner, 2012). Second, some multivariate traits cannot be sensibly reduced to a single measurement. The wing shape we study is a great example of such a multivariate trait. We have good reason to believe that wing shape is important for flight (Ray et al., 2016), but we cannot yet say that any feature, such as wing length or width, is more or less important than any other. Natural selection on wing shape may affect any or all combinations of measurements.

Perhaps most importantly, traits are not inherited in isolation, but are the joint outcome of an integrated developmental process that results in extensive genetic correlations that can have important effects on evolution. The main source of such correlations are the patterns of pleiotropic effects generated by mutational effects. Multivariate studies of inheritance allow pleiotropic effects to be estimated in a rigorous and justifiable manner (Melo et al., 2019). The multivariate breeder's equation, $\Delta \boldsymbol{z} = \boldsymbol{G\beta}$, enables short term prediction of evolutionary responses. Key to understanding how populations respond to selection in the short term requires an understanding of properties of the genetic (co)variance matrix (**G**), and in particular the axis of greatest genetic variation, $\mathbf{g}_{max}$. Studies demonstrate that the direction of $\mathbf{g}_{max}$ influences evolutionary trajectories (Blows and McGuigan, 2015; McGuigan, 2006; Schluter, 1996). The degree to which genetic effects associated with particular variants align to major axes of genetic (co)variance, expressed through **G**, may provide insights into which alleles are most likely to be "captured" by selection (Pitchers et al., 2019).

Due to the polygenic nature of complex traits, including multivariate ones, it is important to consider not only the direction of effect for alleles in a single gene but also

108 correlated effects between genes contributing to the phenotype. Interestingly, initial
109 comparisons of directions of genetic effects among induced mutations in two *Drosophila*
110 *melanogaster* wing development pathways showed only partially correlated effects on wing
111 shape within and between pathways (Dworkin and Gibson, 2006). However, recent work has
112 demonstrated that despite large differences in magnitude, the direction of genetic effects of
113 variants segregating in populations are sometimes similar to those from validation experiments
114 using RNAi knockdown of those same genes (Pitchers et al., 2019). Additionally, Pitchers et al.
115 (2019) demonstrated this shared direction of effect could also be shared between a SNP and
116 RNAi knockdown of other genes in the same signaling pathway, such as those involved with
117 hippo signaling, a key pathway involved with wing growth and morphogenesis (Pan et al.,
118 2018).

119 Pitchers et al. (2019) identified over 500 polymorphisms contributing to wing shape
120 variation in the *Drosophila* genetic resource panel (DGRP). Among these, the hippo pathway
121 was over-represented in SNPs associated with wing shape (Pitchers et al., 2019). The degree to
122 which identified hippo pathway variants reflect allele specific effects, differences in magnitude
123 of genetic effects, and even the large statistical uncertainty associated with genetic effects of
124 small magnitude are unclear. Given common dominance patterns, and the likely non-linear
125 genotype-phenotype relationships of most genetic effects, small to moderate changes in gene
126 function may result in modest phenotypic effects (Green et al., 2017; Melo et al., 2019; Wright,
127 1934). Large effect mutants and many RNAi knockdown studies have moderate to large
128 phenotypic effects that are not reflective of the magnitude of genetic effects of SNPs
129 contributing to phenotypic variance in natural populations.

130 The expression of genetic effects also depends on genetic and environmental context,
131 with gene-by-gene (GxG) and gene-by-environment (GxE) interactions contributing to
132 phenotypic variation. The context-dependence of genetic effects for a multivariate trait has
133 been demonstrated for *Drosophila* wing shape. Variants in *Epidermal growth factor receptor*
134 (*Egfr*), influencing *Drosophila* wing shape are replicable in both lab reared, and wild-caught
135 cohorts (Dworkin et al., 2005; Palsson et al., 2005; Palsson and Gibson, 2004). However, in
136 replication studies, effect sizes of alleles were diminished in both outbred populations and wild

137 cohorts. In the latter case the same variant explained 1/10 of the phenotypic variance explained
138 in the initial study (Dworkin et al., 2005). Interestingly, in a series of experimental crosses
139 among strains, the effects of the SNP were replicable for direction and magnitude in multiple
140 experimental assays and crossing schemes. Despite this, the genetic effect on wing shape from
141 this SNP largely disappeared in one natural population (Palsson et al., 2005). A number of
142 reasons have been proposed for the failure to replicate genetic effects including environmental
143 effects, differences between controlled lab and natural environments (Dworkin et al., 2005),
144 and genetic background (Greene et al., 2009), among others. Because both environment and
145 genetic background likely affect the genotype-phenotype map in a non-linear fashion (Wright,
146 1934), it is important to test observed associations in other experimental contexts.
147 A promising approach to confirm the estimated effects of candidate genetic variants is
148 to test whether they respond to artificial selection in the direction of the inferred effect. This
149 approach is particularly relevant to evolutionary questions, but has rarely been used. In this
150 study, we use artificial selection and bulk segregant analysis (BSA), to replicate and validate
151 SNPs associated with three genes, previously identified in a GWAS of *Drosophila* wing shape
152 (Pitchers et al., 2019); *dachsous (ds), an atypical cadherin involved with hippo signaling;* the
153 transcriptional co-repressor *extra-macrochetae (emc),* and the E3 ubiquitin ligase *neuralized*
154 *(neur)*, involved with Notch signaling. Using the vectors of shape change based on RNAi
155 knockdowns of each gene, we demonstrate that the direction of shape change for these genetic
156 effects is aligned with major axes of natural phenotypic and genetic variation. Using artificial
157 selection based on the direction of shape change defined by RNAi knockdown, we were able to
158 replicate the effects observed for *ds,* but not *emc*, likely due to the available genetic diversity in
159 the population. We then asked if these effects could be replicated in a natural population using
160 a bulk segregant approach, observing little evidence for replication in these samples. We
161 discuss our results in the context of the replicability of genetic effects and the shared direction
162 of genetic effects due to shared developmental processes.
163
164 **Methods**
165 **Source Populations and phenotypic analysis**

166 *Drosophila* strains

167 Phenotype data for the *Drosophila* genetic resource panel (DGRP) was collected for 184
168 strains as part of a GWAS study as described in Pitchers *et al* (2019). Genotype data for these
169 strains was obtained from freeze 2 of the DGRP (Huang et al., 2014). For replication using
170 artificial selection, 30 DGRP strains were used: DGRP-149, 324, 383, 486, 563, 714, 761, 787,
171 796, 801, 819, 821, 822, 832, 843, 849, 850, 853, 859, 861, 879, 887, 897, 900, 907, 911, 913.
172 These strains were selected to increase genetic variation at the *ds* locus (Supplemental Figure 1,
173 Table 1). Reciprocal pairwise crosses between the 30 selected DGRP strains were used to create
174 heterozygotes and these 30 heterozygous genotypes were successively pooled for 4
175 subsequent generations, allowing for recombination. After pooling, the synthetic outbred
176 population was maintained for approximately 47 subsequent generations (allowing for
177 recombination) before the start of artificial selection experiments.

178 For the replication in wild-caught populations using BSA, individuals were collected via
179 sweep-netting from orchards and vineyards in Michigan and after species identification, stored
180 in 70% ethanol. In 2013 and 2014, cohorts were collected from Fenn Valley Winery (FVW13 and
181 FVW14 respectively, GPS coordinates: 42.578919, -86.144936). Additionally in 2014, cohorts
182 were collected from Country Mill Orchard (CMO, GPS coordinates: 42.635270, -84.796706), and
183 Phillip's Hill Orchard (PHO, GPS coordinates: 43.117981, -84.624235). For all collected cohorts,
184 except for the FVW14 collection, only males were used in this study given difficulties
185 distinguishing *Drosophila melanogaster* and *D. simulans* females morphologically. For the
186 genomic analysis of the FVW14 wild caught population (below) we utilized both males and
187 females as the number of individuals was insufficient otherwise. For the collection where
188 females were included in the study, there is no evidence of contamination with *D. simulans* as
189 all dissected wings were classified as *D. melanogaster* using linear discriminant analysis (LDA).
190 LDA was trained using male wings from the collected *D. melanogaster* data set and males from
191 *D. simulans*. There was 100% agreement between the classification of females within each
192 species with our phenotypic classification, indicating that it is unlikely that *D. simulans* females
193 were included in our samples (Supplemental Figure 2).

194 Morphometric Data

195 Landmark and semi-landmark data were captured from black and white TIFF images
196 using the pipeline described in Houle et al. (2003). First, two landmark locations, the humeral
197 break and alula notch, were digitized using tpsDig2 (version 2.16). Wings (Van der Linde 2004–
198 2014 , v3.72) software was used to fit nine cubic B-splines, and manually correct errors. All
199 shape data was subjected to Procrustes superimposition (registration), removing the effects of
200 location, isometric scaling, and and minimizing effects of rotation, , via an iterative least
201 squares approach (Rohlf and Slice, 1990). Generalized Procrustes superimposition (registration)
202 and extraction of 14 landmarks and 34 semi landmarks was done using CPR v1.11 (Márquez
203 2012–2014, Figure 1). Sliding of semi-landmarks utilized minimization of Procrustes Distance as
204 the objective function. Superimposition results in the loss of 4 possible dimensions of variation
205 while semi-landmarks are constrained to vary along one "axis", restraining these points to
206 approximately a single dimension of variation each. This results in a total of ~58 available
207 dimensions of shape variation, that can be summarized using the first 58 Principal components
208 (PCs). Allometry was adjusted for in the analysis by fitting a model for landmark coordinates
209 onto centroid size, and using the residuals from this model (Klingenberg, 2022). By accounting
210 for the allometric component of shape, shape variation associated with size variation can be
211 accounted for (Supplemental Figure 3). For most analyses, 'allometry corrected' shape data
212 were used, with the exception of shape models fit using the Geomorph package in R, where
213 Procrustes landmarks were used and centroid size was included as a predictor in the model.
214 <u>Generation of shape vectors for artificial selection and bulk segregant analysis</u>
215 A panel of shape change vectors was estimated using the progesterone-inducible
216 Geneswitch GAL4, under the regulation of an ubiquitous *tubulin* driver, to drive the expression
217 of RNAi for genes of interest (*ds*, *emc*, *neur*), as previously described in Pitchers *et al.,* 2019.
218 GAL4 expression was induced throughout larval development by adding mifepristone, an
219 analog of progesterone, to the larval food. Knockdown was varied by assaying phenotypes at
220 mifepristone concentrations of 0.3, 0.9, and 2.7 µM, plus a control without mifepristone. Wing
221 shape change associated with knockdown of the gene of interest was estimated using
222 multivariate regression of shape on concentration of mifepristone. Shape change vectors
223 estimated from the RNAi experiments for *ds,emc* and *neur*, were used in this experiment

224 (Figure 1b, Supplemental Figure 4). The magnitude ('length') of the vector measures how much
225 shape change occurs per unit change in mifepristone. In general, vectors of greater magnitudes
226 enable better estimate of direction of effect for shape change. As reported in Pitchers et al.,
227 (2019), the magnitude (l^2-*norm*) of vectors for RNAi knockdown of these genes are 5.5 for *ds*,
228 2.8 for *neur*, and 0.44 for *emc*.

229 Shape data collected as part of a previous study (Pitchers et al., 2019) was used to
230 assess the relationships between shape change vectors from the RNAi titration and $\mathbf{g}_{max}$, the
231 first eigenvector of the **G** matrix estimated from DGRP line means. The effects of sex, centroid
232 size and their interaction were removed using a linear model and these residuals were used to
233 calculate shape score by projecting the data (see Supplemental Figure 5) onto the shape change
234 vector estimated in each knockdown experiment. To assess major axes of genetic variation
235 among DGRP strains, principal component analysis was performed on allometry adjusted model
236 residuals (Supplemental Figure 5B). PCA was done in a similar manner for individuals from the
237 wild caught cohorts. Correlations between the first three eigenvectors ("genetic PCs" including
238 $\mathbf{g}_{max}$), the first three PCs from the wild caught cohorts and the shape scores for *ds*, *emc* and
239 *neur* were calculated (Figure 1a, Supplemental Figure 5). From this, *ds, emc* and *neur* shape
240 change vectors were selected for further experiments given high correlation with directions of
241 natural genetic variation (Figure 1, Supplemental Figure 5). Note, as described below, while *ds*
242 and *emc* were used for artificial selection, due to the similar response between them, we used
243 *ds* and substituted *neur (*for *emc)* for the BSA.

244 <u>Artificial selection of synthetic outbred population</u>

245 The synthetic outbred population resulting from pooling DGRP lines was used as the
246 parent population for artificial selection. Both the *ds* and *emc* artificial selection experiment
247 were carried out with three independent replicates of each "up" and "down" selection regimes,
248 along with unselected control lineages. Each generation, wings of live flies were imaged using
249 the 'wingmachine' system and shape data collected (Houle et al., 2003, Van der Linde 2004–
250 2014 ,v3.72). Shape scores were calculated by projecting the data onto the *ds* or *emc* shape
251 change vector as described above, and the 40 individuals each with highest or lowest shape
252 scores, were selected to found the next generation (Supplemental Figure 5A). For the control

253 lineages, 40 individuals were randomly selected for the next generation within each replicate
254 lineage. Following seven generations of selection, 75 individuals from each lineage were
255 selected for pooled sequencing, described below. The response to selection was evaluated both
256 by computing Procrustes distance (PD) between average shape of wings between generations
257 one and seven, and using shape scores (projections) with a linear mixed effect model allowing
258 for the fixed effect factors of treatment and sex, continuous predictors of centroid size and
259 generation, with third order interactions among these effects. The effect of generation was
260 allowed to vary by replicate lineages ($\mathrm{lmer(ds \sim (CS + Sex + line + gen0)\hat{}3 + (1 +}$
261 $\mathrm{gen0|line{:}rep))}$. Realized heritabilities were estimated separately for up and down selection
262 lineages, from the slope of the regression of cumulative selection differentials on cumulative
263 selection response, averaging over sex and with a random effect of replicate lineage.
264 Wild populations
265 For the BSA, wings for wild caught individuals were dissected and mounted in 70%
266 glycerol in PBS. Images of wings were captured using an Olympus DP30B camera mounted on
267 an Olympus BX51 microscope (Olympus software V.3,1,1208) at 20X magnification. When
268 possible, both left and right wings were dissected, imaged and averaged to calculate an
269 individual's mean shape. For some individuals a wing was damaged so only one wing could be
270 used. Shape was captured as described above. The total number of individuals phenotyped
271 from each cohort can be found in Supplemental table 1.
272 To remove allometric effects in the data, shape was regressed onto centroid size and
273 the model residuals were used for all subsequent morphometric analysis. Only data from males
274 was used to compare shape in wild populations, although, including females from the FVW14
275 population and regressing shape onto centroid size and sex gave equivalent results
276 (Supplemental Figure 6). To test for shape differences between collection cohorts, the effect of
277 centroid size and collection cohort on shape were modeled (procD.lm(shape ~ CS + pop_year))
278 using the procD.lm function in Geomorph v 3.1.3. (Adams and Otárola-Castillo, 2013) and
279 distances between populations were calculated using the pairwise function. To select
280 individuals for sequencing, a 'shape score' was calculated using the method described above.
281 Shape data was projected onto the vector of shape change defined by the *ds* or *neur*

282 knockdowns. The *emc* projection vector was not used for BSA due to the high similarity with *ds*
283 shape change (Figure 1), and the similarity of the selection response. Its inclusion would result
284 in selection of largely the same cohorts of individuals for sequencing for both *ds* and *emc*. As an
285 alternative, we utilized the *neur* shape vector as it was largely uncorrelated with that of *emc*
286 and *ds*, but strongly correlated with natural variation in shape. The 75 most extreme individuals
287 on the shape score distribution, within each wild-caught cohort, were selected for pooled
288 sequencing. Allele frequencies within each population was estimated by sequencing 75 random
289 individuals within each cohort. The difference vector between mean shapes of selected pools
290 (within each population) was used to calculate Procrustes distance (PD) between pools and the
291 correlation of this shape change vector with the selection vector used. An estimate of genetic
292 distances between populations was calculated using allele frequencies (mapping pipeline
293 described below) in the pools of the 75 randomly selected individuals using Bray's distance with
294 the vegdist() function from the vegan package (v2.6-2) in R.

295 Sequencing and Genomic Analysis

296 DNA extractions from pools of selected individuals was performed using a Qiagen
297 DNeasy DNA extraction kit. Library preparation and Illumina sequencing was performed at the
298 research technology support facility at Michigan State University. All library samples were
299 prepared using the Rubicon ThruPLEX DNA Library Preparation kit, without a procedure for
300 automatic size selection of samples. Paired end libraries (150bp) were sequenced using Illumina
301 HiSeq 2500, with each sample (either one pool of 75 individuals in the BSA or one pooled
302 replicate lineage in the artificial selection) being run on two lanes.

303 Reads were trimmed with Trimmomatic (v0.36) to remove adapter contamination and
304 checked for quality using FastQC prior to alignment (Bolger et al., 2014). Trimmed reads were
305 aligned to the *Drosophila melanogaster* genome (v6.23) using BWA-MEM (v0.7.8)(Li and
306 Durbin, 2010). Sequencing replicates of the same biological samples were merged using
307 SAMtools (v1.11). PCR duplicates were removed using Picard with the MarkDuplicates tool (v
308 2.10.3) and reads with a mapping quality score less than 20 were removed using SAMtools (Li et
309 al., 2009). A local realignment around indels was performed using GATK using the
310 IndelRealigner tool (v3.4.46). For artificial selection experiments, reads were merged for all up,

311 down and control selection lines as replicates lineages were independent. For wild cohorts,
312 pools were not merged between populations. mpileup files were created using SAMtools and
313 used for subsequent genomic analysis. Highly repetitive regions of the *Drosophila* genome were
314 identified and subsequently masked in mpileup files using RepeatMasker (v4.1.1) with default
315 settings. INDELs and regions within 5bp of an indel were identified and masked using
316 popoolation2 scripts. Population genetic statistics were calculated using PoPoolation (v1.2.2)
317 and PoPoolation2 (v1.201) (Kofler et al., 2011b, 2011a).

318 For the BSA in the wild-caught cohorts, a modified Cochran-Mantel-Haenszel (CMH) test
319 was used to measure significantly differentiated sites between pools of individuals. Sampling
320 effects were accounted for using the ACER package (v.1.0) in R, assuming $N_e = 10^6$ with 0
321 generations of differentiation between selected pools (Spitzer et al., 2020). To adjust for
322 multiple testing, the p-value was corrected using a Benjamini-Hochberg correction (Benjamini
323 and Hochberg, 1995) with an adjusted alpha of 0.05. For each significant site from the CMH
324 test, using an adjusted p-value cut-off of 0.05, we identified the nearest gene using BEDtools
325 (v2.19.1) (Quinlan and Hall, 2010). In addition, to account for sampling variation, we sampled
326 genomic coverage to 75x for all samples, dropping sites that did not meet this threshold and
327 repeating the CMH test. We confirmed that there was no association between genetic and
328 shape differentiation between populations, and that the populations do not show strong
329 phenotypic differentiation based on either overall shape variation, or shape scores used to
330 identify selected individuals for BSA (Supplemental Figures 3 and 7). There was some variation
331 among populations in overall wing size (Supplemental Figure 8), however we (assuming
332 common allometry) adjusted for allometric effects on shape.

333 For artificial selection experiments, F_{ST} was calculated in 5000bp windows. We chose
334 this window size as it is expected that blocks of LD in the synthetic outbred population will be
335 much larger in comparison to that of the wild caught samples (King et al., 2012a; King et al.,
336 2012b; Marriage et al., 2014). This statistic was used to compare the "up" selected pools to the
337 "down" selected pools to help identify regions of differentiation between selected populations.

338 For the artificial selection comparisons, genes in regions of high F_{ST} were identified by
339 finding overlaps between outlier windows and annotated *Drosophila* genes using

340 GenomicRanges (v1.46.1) in Bioconductor. High F_{ST} was defined as F_{ST} values greater than three
341 standard deviations above the mean. GO terms associated with identified genes were
342 annotated using TopGO package (v2.34.0) (Alexa et al., 2006) in Bioconductor. GO enrichment
343 was then performed to identify those terms overrepresented in the identified list using TopGO
344 and a Fisher's exact test. Over representation of 2 GO terms in outlier windows (hippo
345 signaling, GO:0035329; negative regulation of hippo signaling GO:0035331) were tested using a
346 permutation test that randomly sampled genomic windows from the total windows for which
347 F_{ST} was calculated and the permutation was run 1000 times. The distribution of the ratio of
348 observed to expected genes annotated with the term of interest within randomly sampled
349 regions was compared to the number observed in the data.
350 Verification of *ds* indel in DGRP
351 Sanger sequencing was performed on individuals from a cross between DGRP lines predicted to
352 have the polymorphism (DGRP 195, 28, 96, 48, 59, 801) and those without (DGRP 129, 301, 69,
353 385, 75, 83, 491, 34, 774) crossed to a line carrying a deletion in the region of interest (BDSC
354 24960) to account for potential residual heterozygosity in otherwise inbred strains. DNA was
355 prepared by incubating flies in DNA extraction buffer (1mM EDTA, 25mM NaCl, 10 mM TrisHCl
356 pH 7.5) for 10 minutes, followed by storage at -20 C. PCR application of the region of interest
357 (Forward primer: ggagtacaagttgctcgaac: Reverse Primer: cagatcgtgttccctttagc) using Taq DNA
358 polymerase (Gene DirectX) (PCR mix: 1uL DNA, 1uL forward primer, 1uL reverse primer, 0.5uL
359 10mM dNTPs, 2uL 10x PCR Buffer, 0.1 uL taq, H20 to 20uL). PCR conditions were as follows: 5
360 minutes 95°, (30 seconds 95°, 30 seconds 55°, 30 seconds 72°)x30. Reactions were checked on a
361 gel and cleaned with the GenepHlow™ Gel/PCR Kit (Geneaid). Sanger sequencing reactions
362 were performed by the Mobix Lab at McMaster University. All alignments were created using
363 ClustalOmega (Madeira et al., 2022).
364 Data Availability:
365 All code and processed data needed to complete the analysis is available on GitHub at:
366 https://github.com/DworkinLab/WingShapeBSA/. A static version of the repository is available
367 on figshare (https://doi.org/10.6084/m9.figshare.22141154.v1).

368 All raw sequence data available as part of the NCBI Short Read Archive, BioProject
369 PRJNA936488 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA936488/), with individual
370 sequence accessions SAMN33354503 - SAMN33354634.
371
372 **Results**
373 *dachsous (ds)* shape change is aligned with major axes of genetic and phenotypic variation in
374 natural populations
375 To assess the relationship between shape change vectors and axes of natural variation
376 described in the the DGRP, mean shape vectors were calculated for each DGRP strain, then
377 used in a PCA to summarize axes of variation among strains. Mean shape vectors for each strain
378 of DGRP were projected onto shape change vectors for *ds emc*, and *neur*, defined from the
379 RNAi knockdowns (see Supplementary Figure 5, which visually explains the procedure),
380 generating gene specific "shape scores". Correlations between shape scores for individual DGRP
381 projected onto the shape change vectors (Figure 1, Supplemental Figure 5), and with PC1
382 generated from the DGRP ($PC1_{DGRP}$) strains was estimated ($PC1_{DGRP}$-*ds*: r = -0.56; $PC1_{DGRP}$-*emc*: r
383 = -0.45; Figure 1). The correlation of the DGRP data, projected onto each of the *ds* and *emc*
384 shape change vectors was also correlated (Figure 1, *ds*-*emc*: r = 0.69). This is likely due to the
385 correlation between gene specific shape change vectors themselves (r = 0.65), based on RNAi
386 titration experiments. Projections of the DGRP data onto the vector defining the *neur* shape
387 change is aligned with PC1 (PC_{DGRP}-*neur*: r = -0.69) and PC3 (PC_{DGRP}-*neur*: r = -0.64), indicating
388 this as an important axis of shape variation in this population (Figure 1), that is moderately
389 similar to projections onto *ds* (*ds*-*neur*: r = 0.56) and very similar to *emc* (*neur*-*emc*: r = 0.83)
390 shape change vectors. Interestingly, the strength of the correlation for the DGRP strains
391 projected onto these vectors, differs from the magnitude of correlations for the RNAi titration
392 vector of *neur* with that of *ds* (r = 0.034) or *emc* (0.3). Because of these observed correlations,
393 and previous associations observed (Pitchers et al., 2019) *ds, emc* and *neur* were selected as
394 focal genes for subsequent studies.
395 We also examined the relationship between direction of phenotypic effects with the
396 wild caught cohorts. For these samples, phenotypic variance for shape is due to the joint

397 contribution of genetic and environmental effects. To illustrate the difference in shape variance
398 in wild populations and the DGRP, we calculated correlations between the first three
399 eigenvectors for shape in the DGRP, the combined wild cohorts as well as the CMO cohort
400 alone. We observed low correlations between the DGRP eigenvectors and those estimated
401 from wild populations (Supplemental Table 2). As observed with the DGRP, there is a
402 substantial correlation between projections of shapes of individuals onto the *ds* shape change
403 vector and PC1 (defined by phenotypic variation among wild caught files, PC_{wild}) in most of the
404 sampled cohorts (*ds*-$PC1_{wild}$ PHO: r = 0.78; CMO: r = 0.87; FVW13: r = -0.22; FVW14: r = 0.95,
405 Figure 2, Supplemental Figure 9). In cohorts where the *ds* shape change vector was not
406 correlated with PC1, specifically the FVW13 collection, this vector is correlated with PC2 (*ds*-
407 $PC2_{wild}$ PHO: r = 0.12; CMO r = -0.44; FVW13: r = -0.63; FVW14: r = 0.19; Figure 2;
408 Supplemental Figure 5). The pattern for the wing shape from wild-caught individuals projected
409 onto the *emc* shape change vector was generally similar to that observed for *ds* (Figure 2). We
410 also observe a correlation between *neur* shape change and PC1 in most cohorts (*neur*-$PC1_{wild}$
411 PHO: r = 0.51; CMO: r = -0.051; FVW12: r = -0.95 FVW13; FVW14: r = -0.084; Figure 2;
412 Supplemental Figure 7). As with the *ds* shape change vector, in some cohorts such as the CMO
413 the stronger correlation is between the *neur* shape change vector and PC2 (PHO: r = 0.22; CMO:
414 r = -0.57; FVW13: r = -0.0.059; FVW14: r = 0.85; Figure 2; Supplemental Figure 7). Interestingly,
415 in the CMO cohort, the correlations between the projection of shape data onto the *ds* and *neur*
416 shape change vectors is low (*ds*-*neur*: r = 0.11, Figure 2).
417
418 <u>Multiple loci linked to hippo signaling - including *ds*- respond to artificial selection for *ds* and</u>
419 <u>*emc* shape changes.</u>
420 To examine if variants in *ds* are contributing to shape variation, and independently
421 replicate the findings of the earlier GWAS (Pitchers et al. 2019), we performed artificial
422 selection experiment for wing shape along the *ds* shape change vector, and examined the
423 genomic response to selection. By the final generation of selection, we observed a substantial
424 shape change in both the "up" (females: Procrustes Distance (PD) = 0.039, males: 0.044) and
425 "down" directions (females: PD = 0.022, males: PD = 0.022), compared to the base population

426 at the start of the experiment. In comparison, the shape change among unselected control
427 lineages was much smaller (females: PD = 0.005, males: PD = 0.005) (Figure 3, Supplemental
428 Figure 10). The direction of phenotypic shape change after seven generations of selection was
429 in a similar direction to the *ds* shape change vector (defined by RNAi knockdown) for both the
430 up (females: r = 0.90, males: r = 0.90) and down (females: r = -0.82, males: r = -0.77) selection
431 lineages. Realized heritabilities, averaged over sex and replicate were moderate (Supplemental
432 Figure 11, up = 0.38, 95% CI: 0.25 – 0.50; down = 0.28, 95% CI: 0.24 – 0.50). Hippo signaling,
433 including the effects of *ds*, is often associated with changes in size (Pan, 2007). However, we do
434 not observe a significant change of wing size in our selection lineages in either sex
435 (Supplemental Figure 12). It is possible that with more generations of selection we would have
436 observed a clear change in size, as there is a trend indicating such divergence (Supplemental
437 Figure 12).

438 Genome-wide patterns of F_{ST} were examined between up and down *ds* selection
439 lineages. We observed strong genetic differentiation linked with the *ds* locus (Figure 3,
440 Supplemental Figure 13), along with several other regions in the genome. One of the SNPs in
441 the intron of *ds* (2L:702560), identified in Pitchers et al. (2019) through GWAS, showed the
442 expected pattern of response to selection, with opposing sign in up and down selection
443 lineages, with the SNP going to high frequency in all three up selection lineages (Table 1). It
444 should be noted that this SNP is near a complex polymorphism including an insertion of 18bp
445 that may result in inaccurate genotyping at this locus (Supplemental Figure 14). Gene ontology
446 analysis for genes in regions of the genome with an F_{ST} greater than 0. 345 (three standard
447 deviations from mean F_{ST}), show enrichment for hippo signaling loci (Supplemental Table 3).
448 The top 20 enriched terms are all related to cell signaling and development. Of note is the
449 inclusion of the terms for 'negative regulation of hippo signaling' (GO:0035331), and 'hippo
450 signaling' (GO:0035329) in this list (Supplemental Table 3, Supplemental Figure 13). Using a
451 permutation test we confirmed these results, selecting random sets of genomic intervals equal
452 in size to the number of observed outlier windows, and measured the ratio of genes annotated
453 to the expected number of genes in these regions. The observed value for the terms for hippo
454 signaling (ratio = 4.76) and negative regulation of hippo signaling (ratio = 9.23) were in the

455 upper 99.5% percentile in comparison to the distributions under permutation (Supplemental
456 Figure 15).
457
458 For the artificial selection experiment based on the *emc* shape change vector we
459 observed phenotypic differentiation under artificial selection in both up (females: PD = 0.043,
460 males: PD = 0.040), and down directions (females: PD = 0.021, males: PD = 0.020), with little
461 change in control lineages (females: PD = 0.009, males: PD = 0.008) (Figure 4). The direction of
462 phenotypic change is correlated with the *emc* (RNAi knockdown) shape change vector in both
463 up (females: r = 0.75, males: r = 0.69) and down (females: r = -0.69, males: r = -0.75) directions.
464 Realized heritabilities, averaged over sex and replicate were calculated for both up and down
465 lineages (Supplemental Figure 16, up = 0.38, 95% CI: 0.29 – 0.47; down = 0.28, 95% CI: 0.21 –
466 0.35). Genetic differentiation linked to the *emc locus* was modest following selection, but we
467 again observed striking genetic differentiation linked to *ds* (Figure 4, Supplemental Figure 13).
468 Notably, as seen in Supplemental Figure 1, the site frequency spectrum (SFS) suggests modest
469 allelic variation at the *emc* locus in the synthetic outbred population. Using a three standard
470 deviation cut-off for F_{ST}, we did observe enrichment for various developmental GO terms, but
471 not of hippo signaling terms (Supplemental Table 4, Supplemental Figure 13).
472
473 <u>Bulk segregant analysis in wild caught cohorts does not recapitulate effects of the GWAS or</u>
474 <u>artificial selection</u>
475 Having demonstrated that variants in (or linked to) *ds* respond to artificial selection for
476 wing shape along the *ds* shape change vector, we next wanted to determine whether we could
477 recapitulate these findings with wild caught individuals. In addition to determining whether we
478 can replicate effects in wild cohorts, it provides the opportunity to identify causal SNPs because
479 of low LD generally observed in wild caught *Drosophila*. Wild caught populations introduce
480 considerably more environmental variation for shape along with a different site frequency
481 spectrum for variants contributing to shape variation (and *ds* like shape changes specifically). In
482 particular, it is known that several of the variants that the original GWAS detected in *ds* have
483 low minor allele frequency (MAF) (Pitchers et al., 2019) (Table 2). The SNP at 2L:702560 does

484 appear to be at intermediate frequency but it occurs both directly before and after an indel,
485 making alignment and variant calling in this region challenging (Supplementary Figure 14). We
486 have included the frequencies (Table 2), but these results should be interpreted with caution
487 due to the technical complexities of mapping and variant calling close to indels.

488 As we sampled multiple cohorts of wild-caught flies in different locations and years in
489 Michigan (USA), we wanted to confirm that any phenotypic differentiation among these
490 samples was modest and would not impact genomic analysis for the BSA. We observe modest,
491 statistically significant wing shape differences among cohorts from a Procrustes ANOVA,
492 utilizing permutations of the residuals for the relevant "null" model (Supplemental Table 5; R^2=
493 0.16, F = 351, Z_{RRPP} = 18.3, p = 0.001) (Collyer and Adams, 2018). This appears to be due to
494 differences in wing shape between the PHO population and other populations based on
495 pairwise Procrustes Distances (Supplemental Table 5, Supplemental Figure 3). In a joint PCA
496 including all populations, there is very modest separation between populations using allometry
497 adjusted shape (Supplemental Figure 3). Most relevant to the BSA approach we used, when we
498 project all wild caught individuals onto the *ds* and *neur* vectors, there is no clear separation
499 among sampling locales (Supplemental Figure 7). There is some variation in wing size between
500 populations (Supplemental Figure 8), but this is unlikely to influence downstream analysis as we
501 use size adjusted estimates. There is little evidence of genetic differentiation between
502 populations with the two collections from Fenn Valley Winery separating more on a Principal
503 Co-ordinate Analysis (PCoA)(Supplemental Figure 17) than other sampling locales. There is also
504 no relationship between genetic and phenotypic distances between samples (Supplemental
505 Figure 18). These results suggests that the multiple sampling locales should not influence
506 downstream genomic analysis as individuals used for generating pools were compared within
507 each population, and we observe little evidence for substantial differences among populations.

508 Because there is a single bout of phenotypic selection distinguishing pools for the BSA,
509 changes in shape and allele frequencies are expected to be modest. We observe shape
510 differences between the two pools within each population (PD = CMO: 0.033; PHO: 0.036;
511 FVW13: 0.040; FVW14: 0.041; Supplemental Figure 19). Correlations of the shape difference
512 vectors of the pools (i.e. difference between the two pools created from the extremes along

513 the *ds* shape change axis), and the direction of the *ds* shape change vector used for selection, is
514 high (CMO: 0.94, PHO: 0.79, FVW13: 0.92, FVW14: 0.90).
515 BSA genome scans show little evidence of genetic differentiation linked to the *ds gene*
516 (Figure 5). Across the genome, 15 sites were detected as significantly differentiated between
517 "up" and "down" selected pools based on a CMH test with FDR cut-off of 5% (Figure 5, Table 3).
518 The genes nearest to these sites are not associated with hippo signaling pathways or implicated
519 in the development of the *Drosophila* wing (Table 3). Because PHO had somewhat distinct
520 shape variation from the other populations and had a lower correlation of the difference vector
521 between selected pools and *ds* shape change vector, we repeated the CMH test with this
522 population left out. We observe significant differentiation at 174 sites between "up" and
523 "down" pools (Supplemental Table 6, Supplemental Figure 20). We identified the nearest genes
524 to these sites and GO analysis indicated enrichment for wing development terms, in particular
525 related to Wnt signaling, but not hippo signaling terms (Supplemental Table 6). Importantly, we
526 do not observe differentiation linked to *ds* or any other hippo loci. To ensure that the results we
527 obtained were not due to uneven coverage between samples, we down-sampled genomic
528 coverage to 75x for each sample, dropping sites that did not meet this threshold. Significant
529 differences were detected at 19 sites (Supplemental Figure 21, Supplemental Table 7), but none
530 of these overlapped with those identified using all the genomic data. Two of the significant sites
531 are located in the *dumpy* gene, a gene known to have a role in wing morphogenesis during
532 pupation (Etournay et al., 2015). F_{ST} between selected and random pools within each cohort are
533 generally low (Supplemental Figure 22).
534 In addition to the BSA selection based upon the *ds* shape change, we also selected pools
535 of individuals based on the *neur* shape change vector. We did not use *emc* shape change in this
536 experiment due to the high similarity between the *ds* and *emc* shape change vectors (r = 0.65),
537 and the similar response to selection reported above. We selected the *neur* shape change
538 vector as it is not aligned with *ds*, but does align with directions of natural variation, in wild
539 populations (Figure 1, 2, Supplemental Figure 9). Additionally, there is little relationship
540 between the *ds* and *neur* shape change axis (r = 0.12, Supplemental Figures 7 and 8) in the wild
541 caught cohorts. We observe shape changes between pools of individuals (PD = CMO: 0.027;

542 PHO: 0.028; FVW14: 0.041; FVW13: 0.038, Supplemental Figure 23). There is little evidence of
543 genetic differentiation between *neur* selected pools (Supplemental Figure 24). Only 4 sites were
544 identified as being significantly differentiated between pools and none of these sites are
545 associated with wing development (Supplemental Table 9). When population differentiation
546 between pools within populations is measured using F_{ST}, genetic differentiation remains low
547 across the genome (Supplemental Figure 25)
548
549 **Discussion**
550 The primary goal of this study was to determine whether we could recapitulate genetic
551 effects initially observed through a traditional GWAS using an "inverted" approach: artificially
552 selecting on phenotypes and observing changes in allele frequencies. We observed that shape
553 changes associated with the *ds*, *emc* and *neur* genes were associated with major axes of genetic
554 variation among a panel of wild type strains (DGRP) reared in the lab, and axes of phenotypic
555 variation among wild caught individuals (Figure 1, 2). After observing a strong response to
556 artificial selection along two shape change vectors (*ds* and *emc*), we examined patterns of
557 genomic differentiation and observed substantial changes in allele frequency for markers linked
558 with *ds* itself (figure 3), and markers linked to numerous genes associated with hippo signaling
559 (Supplemental Figures 13 and 15).
560 In contrast, our BSA experiments, using pools of wild caught individuals chosen to be
561 phenotypically divergent on the same shape vectors, did not detect differences in the loci
562 identified in the artificial selection experiments (Figure 5, Supplemental Figure 24). As we
563 discuss in detail below, these seemingly contradictory results are in fact not that surprising.
564 Following artificial selection based on *ds* shape change we observe allele frequency changes
565 not only at *ds* but also linked to a number of other hippo signalling loci (Figure 3, Supplemental
566 Table 3). The previous GWAS study identified a number of loci associated with wing shape
567 variation in the DGRP, however, this approach cannot predict which alleles are causative
568 (Pitchers et al., 2019). In our synthetic outbred population, we maximized variation among
569 haplotype blocks containing many of the candidate SNPs in *ds*, increasing our ability to detect
570 frequency changes at and near the implicated variants. Although LD blocks in the outcrossing

571 population from this study remain large, *ds* variants exist on multiple distinct haplotypes,
572 allowing for an examination of allele frequency changes for each. Of particular interest is SNP
573 2L:702560, previously identified though GWAS (Pitchers et al., 2019) as influencing wing shape
574 variation. It was driven to near fixation in each of the artificial selection lineages (Table 1).
575 Although this polymorphism is annotated as a SNP, this region may contain a complex
576 polymorphism (Supplemental Figure 14), making it difficult to accurately assess genotypic calls.
577 Because of this, the predicted allele frequency in the founding population and allele
578 frequencies in this region may be inaccurate. Previous studies demonstrate the importance of
579 alleles at intermediate frequency in founding populations to those contributing to responses to
580 selection over short timescales (Kelly and Hughes, 2019). If this polymorphism is at a more
581 intermediate frequency in the founding population, it would be more likely to be captured by
582 selection during these experiments. Additionally, haplotype blocks in the initial population are
583 large, and may contain many potential functional variants. However, based on the results of
584 both the current and previous studies, these *ds* variants associated with 2L:702560 are good
585 candidates for functional validation in future work.
586 When selecting on the *emc* shape change vector, which is similar to that of the *ds* shape
587 change, we observe only a modest allele frequency change at *emc*, and a more robust response
588 at *ds* (Figure 4). In hindsight, this is not particularly surprising and there are multiple
589 contributing factors. Given the increased genetic diversity at *ds* compared to *emc* in the
590 founding population, alleles in *ds* may have provided a more accessible genetic target, as
591 selection can only act upon the diversity available in the population. Additionally, if our
592 estimated direction of effects and selection for *emc* (based on RNAi knockdown) was not well
593 aligned with the actual direction of *emc* SNP effects, this could result in weaker selection on
594 variants at the *emc* locus. It is worthwhile pointing out the small magnitude of the *emc* shape
595 change vector (0.44) relative to *ds* (5.5). However, previous work has indicated that there is a
596 relationship between this estimated *emc* shape change vector (from RNAi) and the effect of
597 SNPs in *emc* on shape change (Pitchers et al., 2019).
598 In addition to a response on allele frequency associated with *ds*, our results suggest a
599 response on segregating variation at other hippo signaling loci in the *ds* artificial selection

experiment. Earlier work has suggested that the direction of effects within signaling pathways are inconsistent for alleles of small effect (Dworkin and Gibson, 2006). However, allelic effect sizes in the 2006 study were heterogeneous and may result in direction and magnitude being confounded. In contrast, in both the current and the Pitchers et al. (2019) studies, we estimated the direction of genetic effects by titrating gene knockdown. The strength of this approach is highlighted in the result that segregating variation at multiple hippo loci was selected on (Supplemental Figures 13 and 15). Our finding is consistent with models for the architecture of complex traits that predict that many alleles of small effect will contribute to trait variation with many genes within developmental pathways (Boyle et al., 2017; Wray et al., 2018). This pathway response has also been demonstrated in human adaptation to pathogen resistance (Daub et al., 2013) and high altitude (Gouy et al., 2017). These results are consistent with the expectation that polymorphisms in the same developmental pathway would show correlated phenotypic effects and therefore correlated genomic responses to selection. However, this may not be reflective of all wild caught populations. In this study, we generated a population that had high diversity at *ds,* while these variants are at much lower frequency in natural populations (Table 2). The amount of selectable variation a variant provides, depends on both effect size, a, and variant frequencies, p, as $V_A = 2p(1-p)a^2$. When allele frequencies are near 0 or 1, even variants with large effects will have only a small contribution to short term selection response. Therefore, the outcrossed population we created here is an ideal situation to validate the existence of the measured effects. It is unlikely to be typical of natural populations where functional variants may be rare.

Given the clear and robust response observed in the artificial selection experiment, it may seem surprising that we do not observe allele frequency changes in the BSA using the wild cohorts. Indeed, previous work has demonstrated that variants in *Egfr*, could be replicated in wild caught samples (Dworkin et al., 2005; Palsson et al., 2005) and were also found in genome wide associations (Pitchers et al., 2019). However, there are many explanations for why we may not have been able to detect these allele frequency changes in our experiment. First, the addition of environmental variation to the system introduces additional complications. In the aforementioned example with *Egfr*, the genetic effect of the SNP in wild-caught cohorts was

629 ~10% of the magnitude estimated in lab-reared flies. As discussed previously, the *ds* variants
630 implicated in the previous GWAS study are at low frequency in the natural cohorts (Table 2).
631 Given that natural populations of *Drosophila* are generally large and wing shape is likely under
632 weak selection (Gilchrist and Partridge, 2001), mutation-drift-selection balance may maintain
633 most variation, resulting in low minor allele frequencies at these sites. Because allelic
634 contribution to wing shape are expected to be both rare in wild populations and of small
635 phenotypic effect, we do not expect large allele frequency changes given only one "generation"
636 of selection. Using the approach of ACER (Spitzer et al., 2020) to account for sampling effects,
637 we observe few differentiated sites, and none in the *ds* gene, indicating that BSA may not be
638 well-suited to identify modest allele frequency changes, thus, not particularly effective for
639 polygenic traits. Although our approach was tailored to look for variants that had consistent
640 direction of frequency changes across the four collection cohorts, it is possible that different
641 loci were contributing variation within each cohort. We attempted to address this question by
642 examining allele frequency changes between selected pools within each cohort (Supplemental
643 Figures 22 and 24) but could not identify specific loci contributing to differences within any one
644 population. Previous successful BSA studies identified smaller numbers of contributing loci with
645 few polymorphisms contributing to the trait of interest. For example, in *Drosophila,* a number
646 of melanin synthesis genes contributing to variance in pigmentation between populations were
647 identified using a BSA (Bastide et al., 2013). Pigmentation may represent a relatively 'simpler'
648 genetic architecture (fewer variants of individually larger genetic effect, smaller impact of
649 environmental variation, smaller mutational target size) and if so, this may have enabled the
650 success of the BSA approach with such systems. In the case of wing shape, we know that many
651 alleles of small effect contribute to variation in the trait (Pitchers et al., 2019).
652 Our approach for the BSA was to perform the same phenotypic selection within each of
653 four distinct "populations". It is important to recognize that there was heterogeneity among
654 our populations, not only in allele frequencies, but in environmental variance and potentially
655 *G*x*E*, even though all were caught in locales in lower Michigan. We detected small degrees of
656 phenotypic and genetic differences between cohorts, however these effects are neither
657 correlated with one another, nor related to the *ds* and *neur* shape scores used for selecting

658 individuals (Supplemental Figures 3, 7, 18). The population from the Phillips Orchard (PHO) was
659 phenotypically distinct from the other populations. When we performed the BSA without this
660 population, we observed a larger set of variants associated with shape (Supplemental Figure
661 20), albeit still not showing any effects at *ds* or *neur* genes themselves. One possibility is that
662 the increased number of sites when the PHO sample is removed from analysis represents an
663 unknown statistical artefact we have not identified. However, a more likely explanation is that
664 there are some large unknown environmental influences (*E*), or that the genetic effects show a
665 degree of *G*x*E* (with a specific environment in PHO) that contributed to shape variation along
666 the *ds* direction in this population. Such obfuscating effects have been observed before with
667 the previously discussed *Egfr* example, where the SNP effect identified and validated in multiple
668 contexts (Dworkin et al., 2005; Palsson et al., 2005; Palsson and Gibson, 2004) could not be
669 detected in one natural population, despite being at intermediate frequencies in each sample
670 (Palsson et al., 2005). Importantly, we did detect differentiation at sites associated with
671 developmental processes in the wild cohorts, suggesting that the failure to detect variation
672 linked to *ds* or other hippo signaling loci (Table 3, Supplemental Table 6,7) is not due simply to a
673 lack of power.
674 The response to selection at *ds* and other hippo signaling loci in the artificial selection
675 experiment based on *ds* shape change indicates that this is an important axis of variation for
676 wing shape. Coupled with the alignment of phenotypic effects of perturbations in genes in this
677 pathway with directions of **G** and **P**, this finding may seem to suggest a developmental bias in
678 available variation. However, we caution against such interpretations based solely on the
679 findings in this study. The structure of the **G** matrix strongly influenced our findings as we
680 artificially created a population to maximize genetic diversity at *ds*. When another effect is
681 aligned with *ds* shape change, as in the case of *emc* shape change, we observed the same
682 response at the hippo signaling loci and not at *emc.* Only the genetic diversity in the starting
683 population was available to be selected on so this influenced selection towards the "spiked in"
684 *ds* variants, even if the inferred phenotypic effects of *emc* variants are very similar.
685 Alternatively, the inferred *emc* direction of effects (via RNAi knockdown) may be sufficiently
686 "distant" from true effects of *emc* variants. If this was the case, we were ineffectively selecting

687 for *emc* shape changes. In other cases where single genes are implicated in divergence between
688 multiple populations, such as *mc1r* in mice (Steiner et al., 2007) or *pitx1* in stickleback (Chan et
689 al., 2010), other factors such as low pleiotropy, developmental and mutational constraints and
690 history of selection in the population are used to explain why these genes are so often
691 implicated in evolutionary change (Gompel and Prud'homme, 2009; Martin and Orgogozo,
692 2013; Stern and Orgogozo, 2008). In our case, it is not *ds* itself that is special but rather the
693 orientation of the **G** matrix to align $\mathbf{g}_{\mathbf{max}}$ with the direction of effect for *ds* that shapes our
694 results. Selection acts on variants aligned with the vector of selection (Reddiex and Chenoweth,
695 2021). By varying the orientation of $\mathbf{g}_{\mathbf{max}}$ in the parental population, we would be able to
696 address questions about the repeatability of hippo overrepresentation and if this can be
697 explained by more than just the orientation of **G.**
698 Despite the need for skepticism about the potential for developmental bias influencing
699 directions of variation, the correlated response of sites linked to multiple other hippo signaling
700 genes is intriguing. Coupling of more traditional mapping approaches like GWAS with short
701 term artificial selection provides an additional route to validation and replication of genetic
702 effects. It also suggests that using multivariate data to address the distribution of genetic
703 effects will pay long-term dividends to our understanding of both inheritance and the evolution
704 of multivariate traits.
705
706
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925 **Figure 1. Projections of data onto RNAi shape change vectors are correlated with major axes**
926 **of shape variation among DGRP strains.** (A) Shape change vectors from RNAi titration
927 experiments for *ds, emc* and *neur* were used, and DGRP line means were projected onto these
928 vectors to calculate shape scores. Eigenvectors for the PCA were estimated based on the same
929 DGRP line means. Vector correlations between shape change vectors from RNAi knockdown: *ds*
930 – *emc*: 0.65, *ds* – *neur*: 0.03, *emc* – *neur*: 0.30. (B) Effect of *ds* shape change estimated from
931 RNAi knockdown, effects not magnified. (C) Landmarks (red) and semi-landmarks (blue) used in
932 geomorphic morphometric analysis on a *Drosophila* wing. PCs 1–3 account for 22%, 20% and
933 9% of the overall, among DGRP shape variance.
934
935 **Figure 2. Projections of data onto RNAi shape change vectors are correlated with major axes**
936 **of shape variation in wild-caught *Drosophila*.** Correlations between projection of shape data
937 from CMO population onto *ds, emc* and *neur* RNAi shape change vectors, and the first three
938 eigenvectors from the PCA, calculated from shape data from all samples in the CMO
939 population. PCs 1–3 account for 24%, 18% and 9% of overall shape variance in the CMO
940 population.
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942 **Figure 3. Artificial selection along *ds* shape change vector influences allele frequencies of**
943 **variants at *ds*.**(A) Phenotypic response to selection based on *ds* shape change vector. Only data
944 from females is plotted for ease of visualization. Each replicate of up (squares), control (dots)
945 and down (triangles) selection lineages are plotted (greys). Estimated response to selection
946 shown along red lines. Wing plots represent the effect of selection on shape change between
947 generation one and seven (red, effects not magnified). (B) Genomic differentiation (F_{ST})
948 between up and down selection treatments measured in 5000bp windows. Red line represents
949 the location of the *ds* locus. Grey line represents 3 standard deviations from genome wide
950 mean F_{ST}.
951
952 **Figure 4. Artificial selection along *emc* shape change vector has modest influence on allele**
953 **frequencies at *emc*, but a greater impact at the *ds locus*.** (A) Phenotypic response to selection
954 based on the *emc* shape change vector. Only data from females is plotted for ease of
955 visualization. Each replicate of up (squares), control (dots) and down (triangles) selection
956 lineages are plotted in greys. Estimated response to selection shown along red lines. Shape
957 change between generation 1 and 7 is indicated on the right. Shape effects have been
958 magnified 5x. (B) Genomic differentiation between up and down selection lineages (F_{ST})
959 measured in 5000bp sliding windows. Red and purple vertical lines represent genomic locations
960 of *ds* and *emc* respectively. Grey line represents 3 standard deviations from genome wide
961 mean F_{ST}.
962
963 **Figure 5. Genetic differentiation between pools selected based on *ds* shape change among**
964 **the wild-caught cohorts.** (A) Genome-wide scan for differentiated loci between pools selected
965 based on *ds* shape change vector using the CMH test implemented in ACER. Points in red
966 indicate sites with significant differentiation. Position of *ds* gene in blue (B) Genomic
967 differentiation at *ds* between pools selected based on *ds* shape change vector. No sites are
968 significantly differentiated in *ds*. The large gap in sites is due to a masked region in the genome

969 due to repetitive sequence and poor (syntenic) mapping scores. (C) Shape difference between
970 selected pools of individuals from one representative (CMO) population, with the mean shape
971 of pools represented in black and red.
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993 Table 1. Variants from Pitchers et al. (2019) in *ds* artificial selection experiment. Estimated
994 effect sizes for SNPs are estimated from a GWAS in the DGRP using LASSO regularized
995 coefficients. Average frequency is given with replicate lineage frequencies in brackets.
996 Estimated effect is the l^2-*norm* of shape differences associated with the variant. MAF = minor
997 allele frequency.

Variant	Estimated Effect	DGRP MAF	Estimated MAF in synthetic outcross	Average allele frequency "up" selection	Average allele frequency "down" selection	Average allele frequency "control" selection
2L:655894	0.072	0.44	0.067	0 (0, 0, 0)	0 (0, 0, 0)	0.003 (0, 0, 0.0105)
2L:702560*	**0.159**	**0.056**	**0.06**	**0.995 (1, 0.98, 1)**	**0.446 (0.32, 0.35, 0.67)**	**0.705 (0.69, 0.56, 0.87)**
2L:702798	0.101	0.089	0.1	0.007 (0, 0.0217, 0)	0 (0, 0, 0)	0.005 (0, 0, 0.139)
2L:718623	0.225	0.033	0	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)
2L:718627	0.11	0.033	0	0 (0, 0, 0)	0 (0, 0, 0)	0 (0, 0, 0)

998 * This is a complex polymorphism with linked SNPs and INDELs, in Pitchers *et al* (2019) a SNP in
999 this region was found to be linked. However, the variant calling pipeline used in this work
1000 recognized an INDEL in this region which was used for counting.
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1011 Table 2. *ds* Variants from Pitchers et al. (2019) in wild-caught cohorts used in the present study.
1012 Estimated effect sizes for SNPs are estimated from the DGRP GWAS with LASSO regularized
1013 coefficients. MAF in wild cohorts was estimated from sequenced pools of 75 random
1014 individuals.

Variant	Estimated Effect (Pitchers 2019)	DGRP MAF	Estimated MAF CMO	Estimated MAF FVW13	Estimated MAF FVW14	Estimated MAF PHO
2L:655894	0.072	0.445	0	0	0	0
2L:702560*	0.159	0.056	0.375	0.473	0.485	0.336
2L:702798	0.101	0.089	0.077	0.101	0.044	0.034
2L:718623	0.225	0.033	0.051	0.021	0.044	0.100
2L:718627	0.11	0.033	0.055	0.020	0.046	0.099

1015 * This is a complex polymorphism with linked SNPs and INDELs, in Pitchers *et al* (2019) a SNP in
1016 this region was found to be linked. However, the variant calling pipeline used in this work
1017 recognized an INDEL in this region which was used for counting.
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1044 Table 3. Significantly differentiated variants for *ds* shape change from the wild-caught cohorts
1045 (BSA).

Location	CMH p-value (FDR corrected)	Gene	FlyBase ID	Distance from ORF (bp)
2R:17491270	0.026	*NT5E-2*	FBgn0050104	0
2R17498059	0.034	*CG30103*	FBgn0050103	2061
2R:17515133	0.022	*CG4853*	FBgn0034230	0
2R: 20537878	0.013	*CG13423*	FBgn0034513	0
2R:23601278	0.005	*CG10332*	FBgn0260455	0
2R:23601278	0.005	*IM18*	FBgn0067903	0
2R:23613785	0.013	*Eglp4*	FBgn0034885	0
2R:23613785	0.013	*Eglp2*	FBgn0034883	0
2R: 23646252	0.016	*retn*	FBgn0004795	0
3L:12831924	0.005	*CG10960*	FBgn0036316	0
3L: 20999119	0.022	*skd*	FBgn0003415	0
3R: 21523866	0.013	*CG7956*	FBgn0038890	0
3R: 2559549	0.011	*Pzl*	FBgn0267430	0
X: 14891220	0.013	*Flo2*	FBgn0264078	0
X: 14891220	0.013	*CG9514*	FBgn0030592	0
X:16039731	0.017	*Muc14a*	FBgn0052580	0
X: 793052	0.011	*CG16989*	FBgn0025621	95
X: 9448676	0.034	*mgl*	FBgn0261260	0

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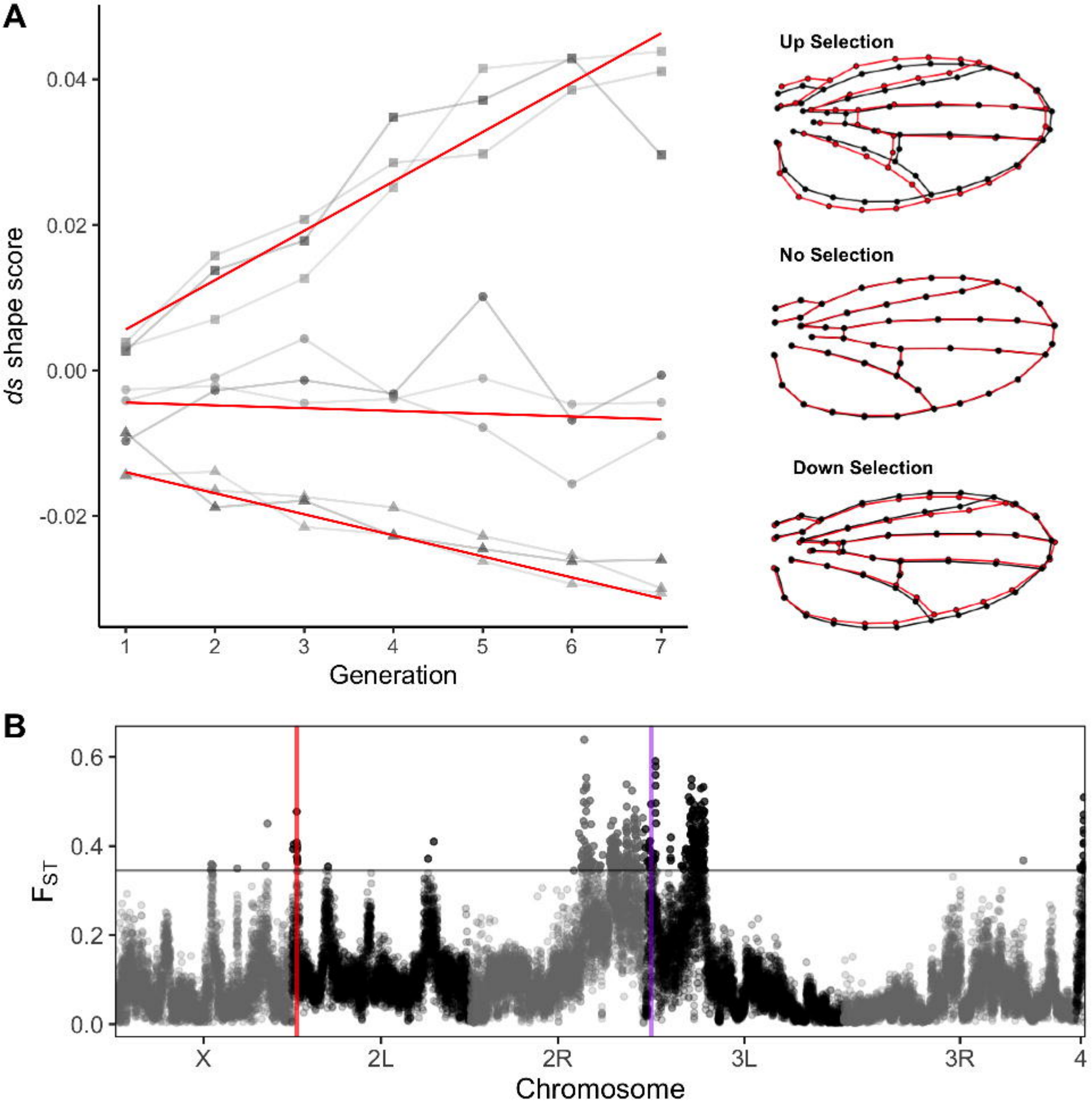
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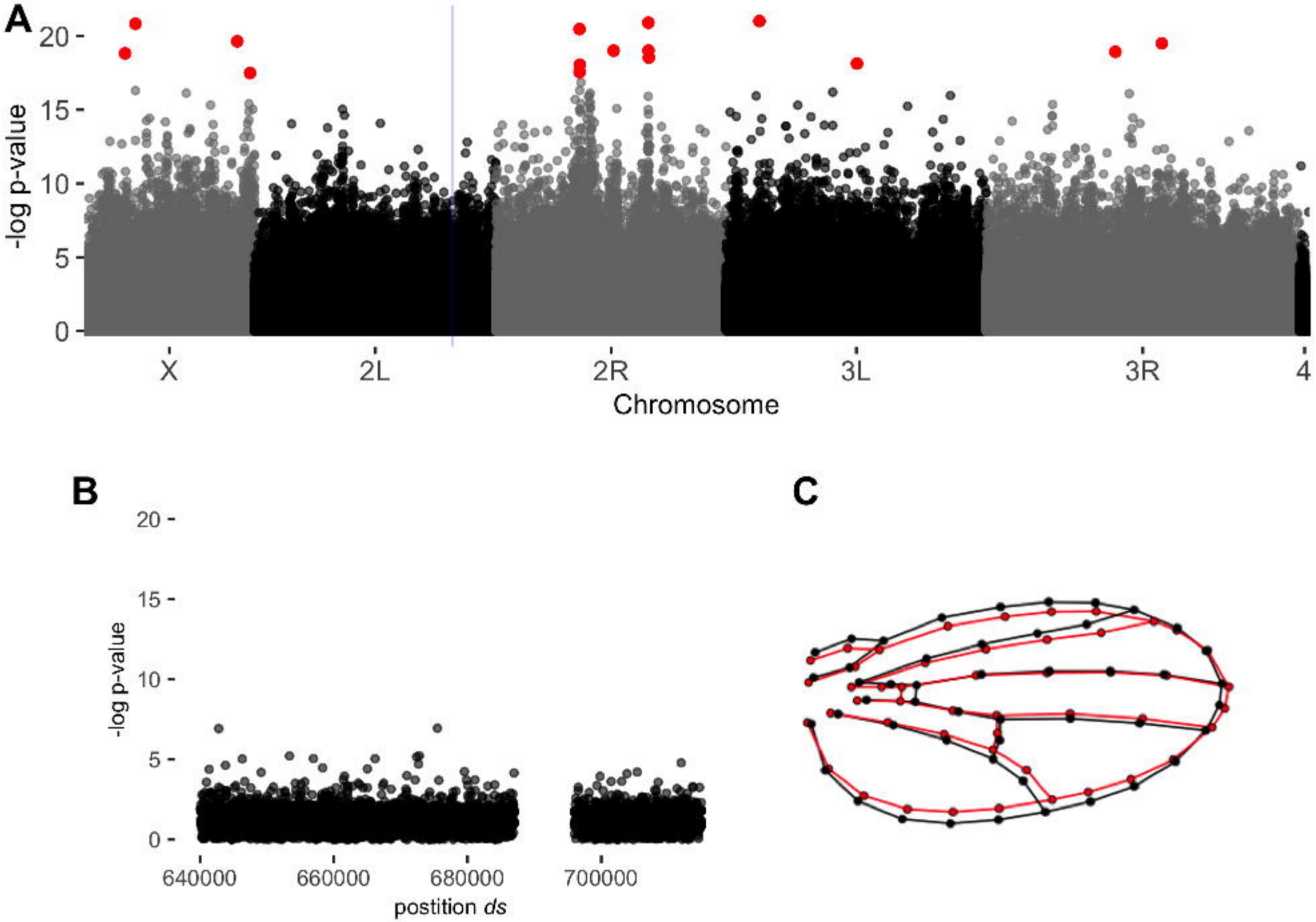
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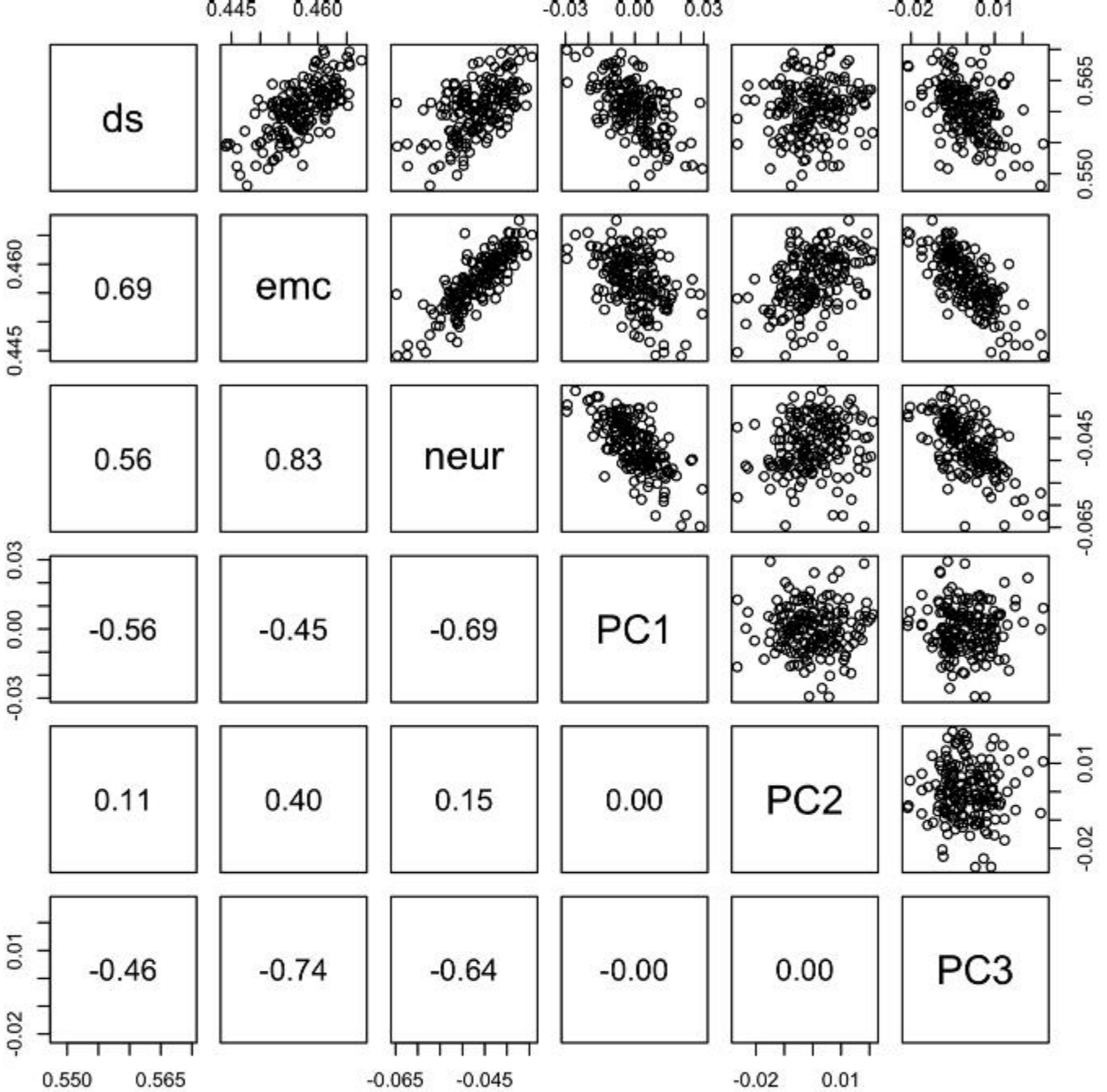
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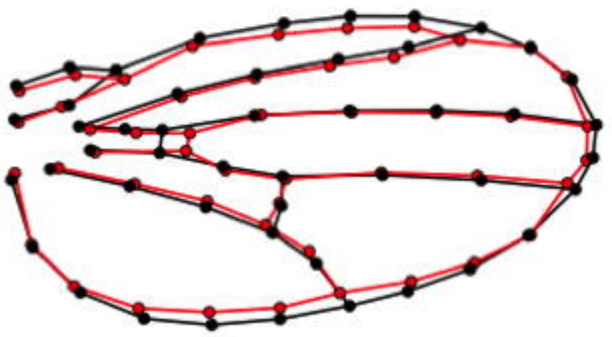
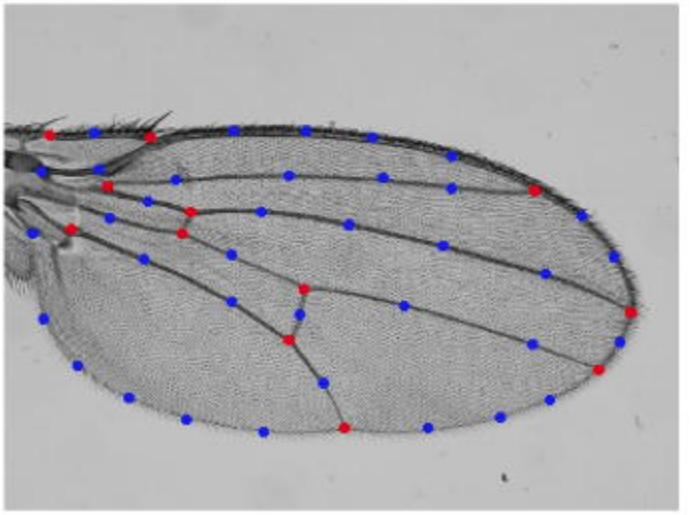
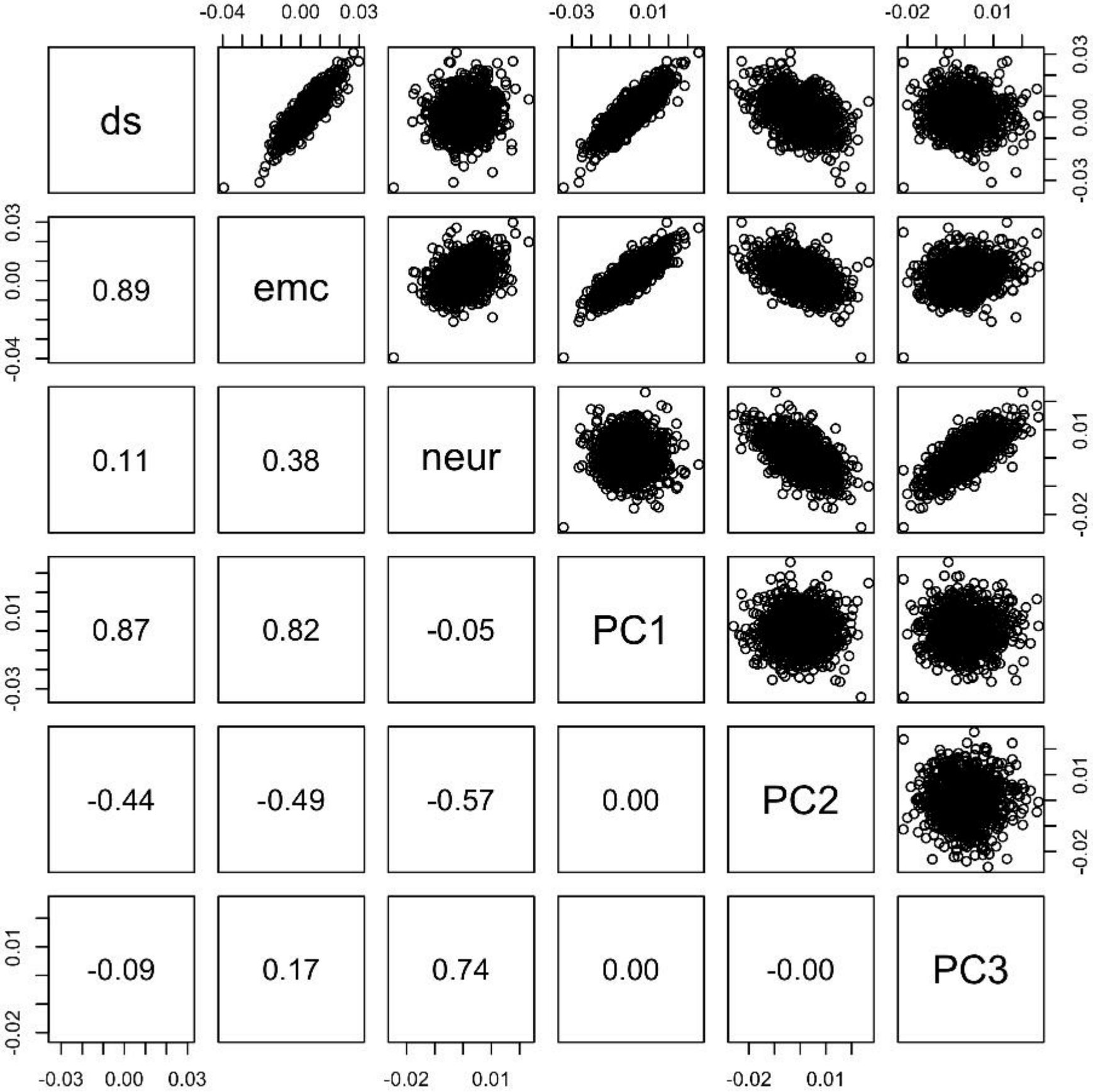
-0.045

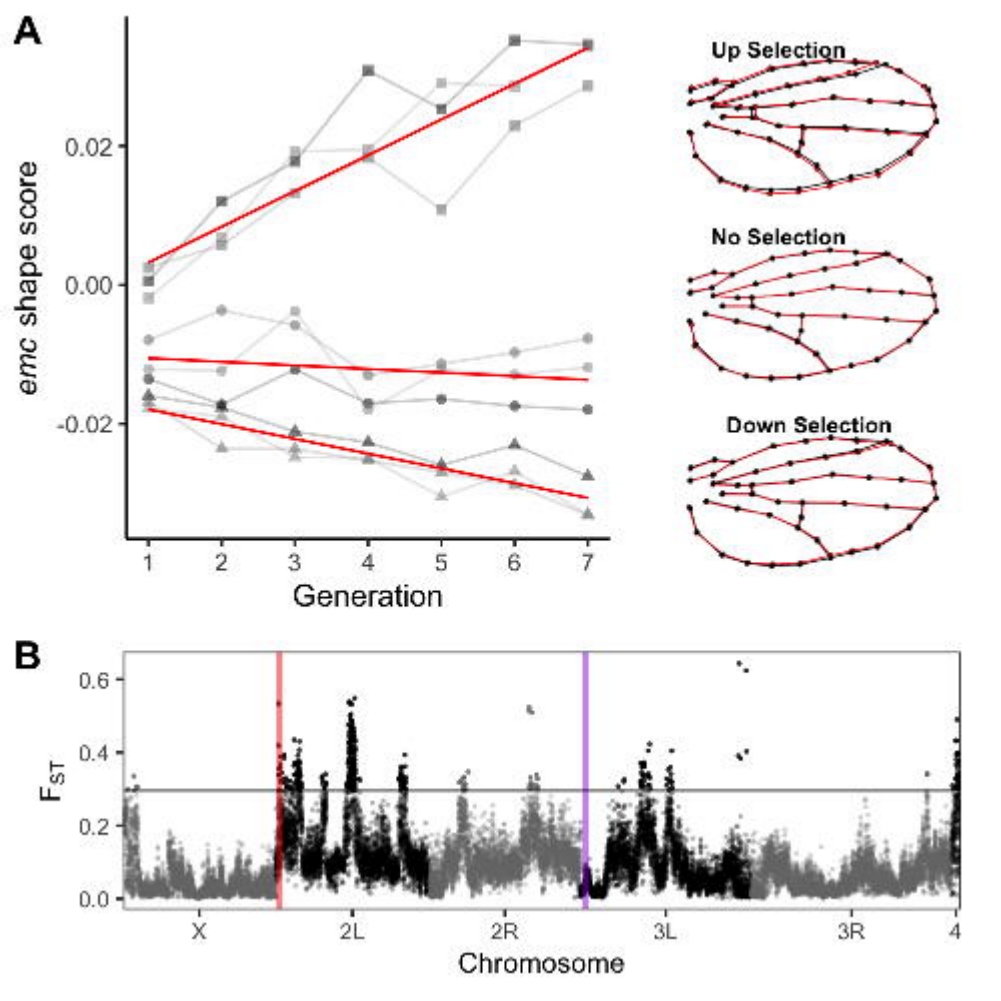
B



C




ds
0.89
emc
0.11
0.38
neur
0.87
0.82
-0.05
PC1
-0.44
-0.49
-0.57
0.00
PC2
-0.09
0.17
0.74
0.00
-0.00
PC3
-0.04 0.00 0.03
-0.03 0.01
-0.02 0.01
0.03 0.00 -0.03
0.03 0.00 -0.04
0.01 -0.02
0.01 -0.03
0.01 -0.02
-0.03 0.00 0.03
-0.02 0.01
-0.02 0.01


A
emc shape score
0.02
0.00
-0.02
1
2
3
4
5
6
7
Generation
Up Selection
No Selection
Down Selection
B
F_{ST}
0.6
0.4
0.2
0.0
X
2L
2R
3L
3R
4
Chromosome