Transcriptomic sexual conflict at two evolutionary timescales revealed by experimental evolution in *Caenorhabditis elegans*

Short Title: Sexual conflict at two evolutionary timescales

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ABSTRACT

Sex-specific regulation of gene expression is the most plausible way for generating sexually differentiated phenotypes from an essentially shared genome. However, since genetic material is shared, sex-specific selection in one sex can have an indirect response in the other sex. From a gene expression perspective, this tethered response can move one sex away from their wildtype expression state and impact potentially many gene regulatory networks. Here, using experimental evolution in the model nematode *Caenorhabditis elegans,* we explore the coupling of direct sexual selection on males with the transcriptomic response in males and females over microevolutionary timescales to uncover the extent to which post-insemination reproductive traits share a genetic basis between the sexes. We find that differential gene expression evolved in a sex-specific manner in males, while in females indirect selection causes an evolved response. Almost all differentially expressed genes were downregulated in both evolved males and females. Moreover, 97% of significantly differentially expressed genes in males and 69% of significantly differentially expressed genes in females have wildtype female-biased expression profile. Changes in gene expression profiles were likely driven through *trans*-acting pathways that are shared between the sexes. We found no evidence that the core dosage compensation machinery was impacted by experimental evolution. Together these data suggest a de-feminization of the male transcriptome and masculinization of the female transcriptome driven by direct selection on male sperm competitive ability. Our results indicate that on short evolutionary timescales sexual selection can generate putative sexual conflict in expression space.

INTRODUCTION

Sexual dimorphism, ubiquitous across multicellular organisms, is rooted in the seeming conundrum that the sexes can be dramatically different yet share largely the same genome [1,2]. On a functional level, this means that gene expression must be tightly regulated in a sex-specific manner [3,4]. On an evolutionary scale, it also means that selection acting differentially on one sex can have an immediate correlated response to selection on attributes of the other sex [5,6]. This correlation underpins the classic Fisherian model of runaway sexual selection, as direct selection acting on a male display trait generates self-reinforcing indirect selection on female preference if they share some form of joint inheritance, either through pleiotropy or linkage [7–9]. Indirect selection can in principle generate a correlated evolutionary response on any number of female traits, however, particularly on polygenic traits for which alleles are more likely to be pleiotropic [10]. For example, males of *Pteridophora alberti*, the King of Saxony bird-of-paradise, have highly elongated feathers equal to several body lengths on either side of their heads. Curiously, females display similar, but much smaller, feathers on their heads, possibly as residual responses to sexual selection in males [11,12]. Much like these birds, opposite-sex perturbation would be expected to be fairly short-lived on an evolutionary timescale, as natural selection against misexpression in the wrong sex leads to heightened sexual dimorphism [13]. The degree to which mating traits have additive genetic covariance, then, determines the opportunity for sexual conflict [14,15]. While the evolution of sexual dimorphism has been extensively studied for macro-level secondary sexual characteristics, such as plumage, territorial behavior, and body size, much less is known at the molecular level, particularly about the coupling between the primary domains of dimorphism: the response to sexual selection at the evolutionary level and the evolution sex-specific gene regulation at the functional level. We address that coupling here with an experimental evolution framework using the nematode *Caenorhabditis elegans*.

In internally fertilizing species, there is an additional layer to the reproductive process to those classically studied under sexual selection, namely post-insemination reproductive interactions. Here the male "trait" of interest can be any cell or protein that affects male fertilization success, while female "preference" is realized in any tissue, cell, or protein that interacts with the male ejaculate to affect female fertilization success. Sexual selection on male reproductive proteins has been inferred through molecular evolution analyses [16–19]. These

73 studies indicate that many seminal fluid proteins evolve rapidly due to sexually antagonistic
74 coevolution, which in turn implies that direct selection is acting on both female and male post-
75 insemination traits. However, identifying the specific female traits with which these male
76 reproductive proteins interact has proved challenging. In this case, transcriptomic analysis can be
77 useful for identifying wide-spread changes in gene expression after mating to uncover polygenic
78 responses to selection within and across generations.

79 Few studies have measured direct selection on male post-insemination traits and the
80 correlated response in females in a similar manner to male display and female preference traits.
81 We previously used experimental evolution to select for increased sperm competitive ability in
82 *C. elegans* males [20]. Experimental evolution provides an opportunity to reproducibly isolate
83 directional selection on male characteristics from the potentially correlated responses in females
84 using timescales when the opportunity for sexual conflict is likely to be highest. We found that
85 after 30 generations of evolution, males showed a strong, rapid response to post-insemination
86 selection at both the phenotypic and genomic levels [20]. Specifically, post-insemination
87 reproductive success increased by greater than four-fold in the evolved populations. This
88 reproductive fitness increase was underlain by a polygenic response of 82 significance peaks
89 represented by 57 genes, 10 intergenic regions, and 15 putative pseudogenes. Together we found
90 that when the androdioecous *C. elegans* mating system is genetically engineered to reflect the
91 ancestral diecious mating state, there is overwhelming selection to improve maleness.

92 Here we test if this strong, direct selection acting on male sperm competitive ability had
93 an indirect impact on females. Specifically, we quantify the impact of direct post-insemination
94 sexual selection on the transcriptomes of mated ancestral and evolved males and compare how
95 selection impacts the transcriptome relative to the genome. Additionally, we examine the
96 transcriptomes of mated females using a fully factorial crossing design of ancestral and evolved
97 individuals to determine: i) whether strong, direct selection on sperm competition generates a
98 response in females, and if so, ii) is the female response evolved or plastic, and iii) is the female
99 response indicative of sexual conflict.

100 RESULTS

101 A strong-inference framework for expression evolution

102 We selected four experimental evolution replicates that evolved under direct selection on sperm
103 competitive ability for 30 generations to test for changes in the transcriptional profile of mated

104 males and mated females. Ancestral and evolved males were crossed with the corresponding
105 generation of ancestral or evolved females to assess how direct selection on males altered the
106 male transcriptome. For females, we used a full factorial crossing design of ancestral and
107 evolved females mated with ancestral and evolved males to determine if the female
108 transcriptome changes and whether those changes were a result of selection (direct or indirect) or
109 female plasticity. Populations of individuals for each cross were mated for 48 hours after which
110 individuals of the focal sex were isolated for transcriptomic analysis. The $ancestor_F$-$ancestor_M$
111 cross represents the baseline transcriptomic profile of both males and females after mating before
112 experimental evolution occurred. The $evolved_F$-$evolved_M$ cross represents the transcriptomic
113 profile of males and females after 30 generations of evolution. If direct sexual selection on males
114 had no impact on a gene's expression level, then there would not be differential expression
115 between the $ancestor_F$-$ancestor_M$ and $evolved_F$-$evolved_M$ cross for that gene. In males, such genes
116 would not contribute to the phenotype under selection, namely sperm competitive ability. In
117 females, such genes would have no genetic covariance between females and males.
118 If instead, in females, a gene is differentially expressed between the $ancestor_F$-$ancestor_M$
119 and $evolved_F$-$evolved_M$ crosses, then females are responding to the sexual selection exerted on
120 males. If the female transcriptional changes are a plastic response to increased sperm
121 competitiveness, then male generation should be a major predictor, that is females of the
122 $evolved_F$-$ancestor_M$ cross should have expression profiles similar to the $ancestor_F$-$ancestor_M$
123 cross and females of the $ancestor_F$-$evolved_M$ should have expression profiles similar to the
124 $evolved_F$-$evolved_M$ cross. Alternatively, if female transcriptional changes are a result of either
125 direct selection in females induce by sperm competitiveness or indirect selection generated by
126 genetic covariation between the sexes, then the expectation is that ancestral females will have
127 similar expression profiles and evolved females will have similar expression profiles, regardless
128 of male generation.

129 **Direct selection on males repeatably downregulates the evolved male transcriptome**

130 We analyzed changes in male gene expression for each experimental evolution replicate
131 separately (Fig S1, Fig S2). Principal component analysis (PCA) was performed to compare
132 samples basted on male generation (*i.e.*, G0 ancestor or G31 evolved). In each replicate, the first
133 principal component partitioned samples by male generation and explained 68% to 82% of the
134 total variance in gene expression (Fig S1). Thus, there was a strong effect of direct selection on

135 the male transcriptome. Differential gene expression analysis showed an overall signature of
136 downregulation with 50% (Replicate B2) to 91% (Replicate A3) of genes being downregulated
137 in evolved males. However, the degree of overlap between significantly differentially expressed
138 genes (DEs) varied greatly among replicates (Fig S3A). Ninety-eight percent of DEs in Replicate
139 A3 were significant in at least one other replicate, while only 60% of DEs in Replicate A2 were
140 shared with at least one other replicate. Replicate A2 also had the most DEs (n = 2,181) and
141 showed the strongest increase in post-mating reproductive success [20], indicating replicate-
142 specific variation in the transcriptional response.

143 Differential evolution between experimental evolution replicates is not surprising and
144 could be cause by multiple processes, including selection acting on different segregating genetic
145 variants and genetic drift. To focus on the consistent and repeatable gene expression changes that
146 are most likely linked with the phenotype under selection, we combined all experimental
147 evolution replicates. The final combined dataset in males consisted of three independent
148 $ancestor_F$-$ancestor_M$ crosses, and 12 independent $evolved_F$-$evolved_M$ crosses.

149 PCA again clustered samples by male generation, such that all ancestral males clustered
150 and all evolved males clustered (Fig 1A). The first principal component explained 42% of the
151 variation in total gene expression and separated males by generation. The combined analysis,
152 therefore, supports the individual replicate analysis and shows a strong effect of direct selection
153 on the male transcriptome. We analyzed differential gene expression using a generalized linear
154 model framework using male generation and correcting for average differences among
155 evolutionary replicates. Differential expression analysis showed 589 genes to be significantly
156 differentially expressed based on male generation of which 125 had at least a two-fold change in
157 expression (Fig 1B; File S1). All but 18 DEs were represented in individual replicate analyses,
158 which indicates that the transcriptional changes identified are likely due to selection (Fig S3B).
159 Replicate did not contribute to significant differential gene expression (File S1).

160 Only five genes were significantly upregulated (mean fold increase = 1.0) in evolved
161 males relative to ancestral males. They are located on autosomes and do not have ontology terms
162 that clearly connect them to reproduction. The other 584 genes (99%) were downregulated (mean
163 fold decrease = 1.5) in evolved males relative to ancestral males. The widespread signature of
164 downregulation is not an artifact of biased count distribution (Fig S4A). Rather, the genes which
165 are highly upregulated in evolved males tended to occur in specific experimental evolution

166 replicates, highlighting the importance of multiple levels of replication when identifying
167 consistent patterns of selection. To determine if SNPs in the coding region or the *cis*-regulatory
168 region of these genes were responsible for the changes in expression, we examined allele
169 frequencies in our previously published genomic mapping data for these populations [20]. None
170 of the DEs had significant allele frequency changes in the coding region. Two genes (*apn-1* and
171 *apa-2*) had SNPs in the *cis*-coding region, however, none of these SNPs showed a significant
172 allele frequency change over the course of experimental evolution (File S2). Together, these data
173 indicate that direct selection on males is downregulating the male transcriptome through *trans*-
174 acting pathways.
175 To assess the functional pathways in which these downregulated genes were involved, we
176 examined their associated gene ontology (GO) terms and KEGG Pathways. GO enrichment
177 analysis identified 20 ontology terms that were significantly enriched, many of which were
178 related to some form of binding: DNA binding (n = 4), RNA binding (n = 2), and protein binding
179 (n = 1) (File S3). Eight KEGG pathways were significantly enriched, including four in the
180 replication and repair class, three in the metabolism class, and one in the endocrine system class
181 (File 3).

182 **Female gene expression changes correlate with males based on female generation**

183 First, to assess variation in the evolutionary response, we analyzed changes in female expression
184 for each experimental evolution replicate separately. PCA clustered females by generation in
185 each replicate (Fig S5). The first principal component explained 30% to 46% of the total
186 variance in gene expressed based on female generation alone. Male generation did not contribute
187 to variance partitioning in the first two principal components. Replicated B3 had two outlier
188 transcriptome replicates, which were censored in the downstream analyses (Fig S5). Differential
189 expression analysis supported that female generation alone determined expression changes as the
190 male term did not identify DEs (Fig S6). Female DEs recapitulated the male signature of
191 downregulation with 92% to 96% of DEs being downregulated in evolved females. The majority
192 of DEs overlapped with at least one other experimental evolution replicate, suggesting less
193 replicate-specific evolution in females than in males (Fig S3C).
194 Although the separate analysis of each replicate helps to highlight variance in evolution
195 response, to more formally address evolutionary changes in the transcriptome that are repeatable
196 across replicates, we used a combined analysis after correcting for among-replicate variance. The

197 final censored, combined dataset in females therefore consisted of three independent ancestor$_F$-
198 ancestor$_M$ crosses, 12 independent ancestor$_F$-evolved$_M$ crosses, 11 independent evolved$_F$-
199 ancestor$_M$ crosses, and 11 independent evolved$_F$-evolved$_M$ crosses. PCA of the full dataset again
200 clustered samples by female generation along the first principal component, which explained
201 28% of the total variance in gene expression levels (Fig 1B). Male generation did not contribute
202 to variance partitioning in the first two principal components, which captured 46% of the total
203 variance. Thus, the combined PCA supports the individual replicate analyses and indicates that
204 the female response is evolved rather than a plastic response based on male generation.
205 To formally examine potential differences of ancestral and evolved males on the female
206 transcriptional response, as well as the influence of evolutionary response to heightened sperm
207 competition of the females themselves, differential expression for individual genes was analyzed
208 using a generalized linear model using female and male generation, correcting for average
209 differences among evolutionary replicates. Neither male generation nor replicate contributed to
210 significant changes in gene expression (Fig 1D; File S1). Rather, female generation determined
211 differential gene expression, again demonstrating an evolved expression response (Fig 1D). One
212 hundred genes showed significant differential gene expression of which eight genes had at least a
213 two-fold change in expression (Fig 1D). Only six genes were significantly upregulated in
214 evolved (mean fold increase = 0.7) females relative to ancestral females. Five of these genes are
215 uncharacterized in function. The other 94 DEs were downregulated (mean fold decrease = 1.3) in
216 evolved females relative to ancestral females (Fig 1D). Again, widespread downregulation is not
217 an artifact of biased count distribution (Fig S4B). None of the significantly differentially
218 expressed genes had significant allele frequency changes in the coding region or the *cis*-
219 regulatory region (File S1). Thus, changes in gene expression were again likely regulated
220 through *trans*-acting pathways. No GO terms or KEGG pathways were significantly enriched in
221 female DEs.

222 **Indirect selection on females drives transcriptome evolution**

223 Since the female transcriptome response is evolved, we next examined whether the changes in
224 female expression were due to direct selection in response to increased sperm competitiveness or
225 indirect selection as a correlated response to the direct selection acting on males. To this end, we
226 examined at the correlation in differential gene expression changes between the sexes. Genes that
227 were only significantly differentially expressed in males (*i.e.*, unique male DEs) were not

228 correlated with female changes in gene expressed (n = 565, p = 0.27, adjusted R^2 = 0.0004; Fig
229 2). Rather, these genes changed expression in males alone. This widespread male-specific
230 transcriptomic response indicates that direct selection on males predominantly changed the male
231 transcriptome in a sex-specific manner.

232 Genes that were only significantly differentially expressed in females (*i.e.*, unique female
233 DEs) were, however, significantly correlated with corresponding changes in male expression (n=
234 76, $F_{4,74}$ = 21.3, p < 0.001, adjusted R^2 = 0.21). Therefore, these genes are largely being
235 downregulated in females and males, though not significantly so in males (Fig 2). This
236 correlation holds when all female DEs – unique and shared between the sexes (n = 24) – are
237 included ($F_{1,98}$ = 42.16, p < 0.001, adjusted R^2 = 0.29). Together, these data support that indirect
238 selection on females was driving changes in the female transcriptome as female expression
239 changes were not independent from those in male, while male expression changes occurred in a
240 sex-specific manner.

241 **Correlated selection de-feminizes the transcriptome of males and females**

242 We hypothesized that selection was driving this widespread downregulation via either female-
243 biased or non-sex biased genes. In either case, the transcriptomic effect would be a less female-
244 like and a more male-like transcriptome to support the increase in male reproductive fitness. To
245 test this hypothesis, we examined the relationship between changes in gene expression after
246 experimental evolution and sex-biased expression in wildtype females and males [21]. The
247 majority (n = 571) of male DEs showed some degree of female-biased expression in wildtype
248 individuals (Fig 3A). A strong negative correlation exists between wildtype sex-bias and gene
249 expression changes in males, such that more female-biased genes in wildtype were more strongly
250 downregulated after experimental evolution regardless of whether the gene was located on an
251 autosome or the X chromosome ($F_{1,568}$ = 1562, p < 0.0001, adjusted R^2 = 0.73). The reduction in
252 female-bias indicates a de-feminization of the male transcriptome.

253 Similarly, the majority (n = 69) of female DEs also showed some degree of female-biased
254 expression in wildtype individuals (Fig 3B). Interestingly, 67 of the downregulated genes (81%)
255 were located on the X chromosome, of which 57 were female-biased (Fig 3B). There is a
256 negative relationship between sex-biased expression and differential gene expression on the X
257 chromosome, such that more female-biased genes had a greater fold decrease in expression ($F_{1,65}$
258 = 12.4, p < 0.001, adjusted R^2 = 0.15). This relationship suggests a pleiotropic effect in a *trans*-

259 regulatory pathway impacting regulation of X-linked genes. No relationship exists between
260 autosomal genes and differential gene expression ($p = 0.14$), though the low number of
261 autosomal genes likely makes this comparison under-powered. Together, these data support a
262 correlated expression response in females and indicate a skew towards a less female-like
263 transcriptome in evolved females.

264 Significantly more DEs are located on the X chromosome in females than in males ($\chi^2 =$
265 319.5, d.f. = 1, $p < 0.0001$) (Fig S7). One major *trans*-regulatory pathway that could explain the
266 correlation between downregulated genes and the X chromosome is dosage compensation. In
267 *Caenorhabditis* nematodes, hermaphrodites/females have two copies of the X chromosome, each
268 of which are partially downregulated to match the dosage of the single X chromosome in males
269 [22]. To determine if whole-scale changes in dosage compensation are responsible for the
270 downregulated pattern in evolved females, we correlated differential gene expression with gene
271 expression levels for dosage compensated and non-dosage compensated X-linked genes [23].
272 Only 31 ($n_{male} = 2$, $n_{female} = 20$, $n_{shared} = 9$) of the significant genes located on the X chromosome
273 were present in both data sets (Fig. 4). Of these, seven were dosage compensated in wildtype
274 hermaphrodites/females (XX individuals) and three were dosage compensated in both sexes.
275 These genes were further downregulated in evolved individuals, particularly in females,
276 potentially suggesting an expression level lower than males. Nineteen genes in total were not
277 dosage compensated in wildtype hermaphrodites/females, but were downregulated in evolved
278 females by on average 1.4-fold. Similarly, the mean fold decrease in wildtype expression in
279 males (XO individuals) for these same genes is 1.9-fold. Eight genes in total were not dosage
280 compensated in wildtype males and were downregulated in evolved males by an average 2.4-
281 fold. While not definitive, these results point to a potential breakdown in proper dosage
282 compensation accompanying widespread transcriptome downregulation.

283 The core dosage compensation genes *sdc-2*, *sdc-3*, *dpy-27*, and *dpy-30* did not have
284 significant changes in gene expression after experimental evolution (File S1) nor were there
285 significant allele frequency changes at or near these genes [20]. Additionally, there were no
286 significant allele frequency changes at the *rex* or *dox* binding sites, indicating that the dosage
287 compensation machinery itself is likely not the cause of widespread downregulation in evolved
288 females. Therefore, alternative pathways must be involved in this de-feminization of the male
289 and female transcriptomes resulting from direct sexual selection acting on males.

290

291 **DISCUSSION**

292 Sexual selection acts in a sex-specific manner yet can have an indirect evolutionary response in
293 the other sex due to genetic associations created by their sharing the same genome (apart from
294 heterogametic sex chromosomes, when present). When sexual selection acts on one sex, the
295 other sex can be pulled away from their sex-specific optimum as a correlated response to
296 selection, generating conflict between the sexes. Here we explored how the transcriptome of
297 newly evolved males responded to direct sexual selection on sperm competitive ability [20] and
298 whether this male-specific selection generated indirect selection on females by quantifying the
299 ancestral and evolved transcriptomes of males and females mated with different experimental
300 evolution generations.

301 Genes with significant expression changes between ancestral and evolved males were
302 almost exclusively downregulated. Moreover, most of these genes are female-biased in wildtype
303 individuals. These results suggest that the evolved male transcriptome moved away from a
304 female-like state. This transcriptomic response supports our previous fitness data [20] and
305 indicates that over the course of experimental evolution these newly derived males improved
306 their reproductive ability by becoming more like males under obligate male-female (dioecious)
307 mating. This highlights the first, and fairly ancient, time horizon for sexual conflict revealed by
308 our data. The transition from the ancestral dioecious mating system within *C. elegans* has led to
309 patterns of gene expression that favor hermaphroditic function—which is essentially very
310 female-like—over male-specific function. Selection for increased male function generated in the
311 populations analyzed here [20], as well as that observed in other studies [24–26], leads to a rapid
312 improvement in male reproductive traits such as sperm size and competitive ability. Our results
313 illustrate that these macro-phenotypic effects are reflected directly in the transcriptome via a shift
314 in the balance of expression-level conflict away from female-biased function.

315 Further, as a byproduct of selection for improved male fitness, females displayed
316 widespread concomitant signature of downregulation in the evolved female transcriptome. This
317 represents sexual conflict on the second time horizon. Selection on regulatory elements that
318 allow enhanced male performance leads to a short-term correlated response to selection on the
319 female transcriptome as well, making it more male-like. Whether we might expect sex-specific
320 transcriptional regulation to rebalance itself over time depends on the specific fitness impacts of

321 these transcriptional changes on females, which are difficult to assess here. Overall, the results
322 indicate a direct de-feminization of the male transcriptome and an indirect masculinization of the
323 female transcriptome generated by direct selection on male fertilization success. Since gene
324 expression levels impact trait function and, in turn, fitness, short-term sexual conflict was likely
325 generated as a byproduct of direct sexual selection in males as female expression was pushed off
326 its global optimum. This give and take is likely to be an important feature structuring
327 transcriptional regulation in many male-female species.

328 The factorial female mating design used here allows for changes in gene expression to be
329 categorized as a plastic response to increased sperm competitiveness in males or an evolved
330 response in the females themselves. If females were predisposed to increased sperm interactions,
331 then male generation should have been a major contributor to gene expression changes. Such a
332 scenario is analogous to a pre-existing bias model of sexual selection [27] and can lead to direct
333 selection on female preference/resistance [14,15]. However, we found no evidence that male
334 evolutionary history impacted differential gene expression. Rather, all expression changes were
335 determined based on whether a female was from the ancestral or evolved populations, indicating
336 that differential gene expression was a result of indirect selection on females during experimental
337 evolution. Under this scenario optimal gene expression differs between the sexes, such that direct
338 selection optimizing the male transcriptome generates an indirect response in females due to a
339 between-sex genetic covariance in expression [6,13,28]. For our post-insemination model, selection
340 was directed on sperm competitive ability. Since selection on males generated a polygenic
341 response and the expression changes observed were likely due to *trans*-acting pathways, it is
342 possible that multiple reproductive trait may be impacted.

343 Interestingly, there was a nearly complete lack of overlap between the genic changes
344 identified in Kasimatis et al. (2022) [20] and the expression changes characterized here. One
345 potential reason for this discrepancy is that the genic changes were altering expression of
346 upstream transcription factors that are expressed earlier in development. Kasimatis et al. (2022)
347 [20] identified genomic changes in the coding sequence of or nearby intergenic region of four
348 transcription factors. Of particular note is the transcription factor *sea-1*, which is involved in sex
349 determination and dosage compensation through hypoactivation of the X chromosome [29].
350 Genomic analysis identified seven SNPs with a significant allele frequency change located
351 within a 30 base pair region of intron 2 [20]. It is possible that this region changes regulation of

352 *sea-1* itself or alters splicing in a way that increase the hypoactivation function of *sea-1* and thus
353 is responsible for the widespread downregulation of X-linked genes.
354 The majority of downregulated genes in females were located on the X chromosome.
355 Because males are the heterogametic sex in *C. elegans*, alleles affecting gene expression on the
356 X chromosome are directly exposed to selection without any potential shielding of an alternative
357 allele due to dominance, as would occur in heterozygous females. Thus, selection can be more
358 effective on X-bearing genes, especially when there is sex-specific selection [30–32]. The
359 response we observe here is thus consistent with the dominance theory of the rapid evolution of
360 sex chromosomes and their role in speciation [30,33].
361 Despite the preponderance of signal on the X chromosome, we found no evidence that
362 mutations in the core dosage compensation pathway were the cause of this widespread
363 downregulation. Downregulation of both dosage compensated and non-dosage compensated
364 genes suggests a potential dysregulation of dosage compensation. These changes further support
365 a less wildtype hermaphrodite/female-like state. Wildtype dosage compensation levels may not
366 represent an expression level that is optimal for both sexes [34–36]. Sexual conflict over dosage
367 compensation creates an obvious additional, but perhaps underappreciated, layer to
368 understanding the evolution of sexual dimorphism across the genotype-phenotype-fitness
369 landscape.
370 Previous experimental evolution studies suggest that male-limited evolution over tens of
371 generations can decrease female fitness [37–39]. Conversely, female-limited or reduced sexual
372 selection evolution tends to increase female fitness with little to no effect on male fitness [40–
373 42]. These previous studies therefore suggest that sex-specific selection acting through males
374 may have a higher potential to generate sexual conflict. However, this previous work has been
375 blind to the traits under selection and therefore the genetic architecture underlying the sexual
376 conflict response. Our work to bridge these layers of the genomic, transcriptomic, and fitness
377 responses suggests a Red Queen scenario [43,44] relating genome evolution and transcriptional
378 dimorphism during post-insemination interactions. Specifically, on microevolutionary timescales
379 strong sexual selection on males can rapidly improve "maleness" at the genomic and phenotypic
380 levels [20] as well as the transcriptomic level, as shown here. In turn, this selection on males
381 pushes females away from their fitness optimum through indirect selection [45,46]. Females may
382 then respond through direct selection, which creates a macroevolutionary picture of a stable

383 female-like state. Thus, examining the functional layers through which sexual dimorphism arises
384 is critical for understanding the context in which sexual conflict can occur.
385 Sexual selection drives the evolution of some of the most elaborate phenotypic variation
386 and complex behaviors. We show that sexual selection is just as powerful an evolutionary force
387 on post-insemination traits. In particular, on microevolutionary timescales, females may not have
388 the opportunity to counter the effects of indirect selection leading to females being pulled away
389 from their optimal state. Given the seemingly high degree of pleiotropy between female and
390 male reproductive networks, sexual conflict may evolve very rapidly in populations experiencing
391 strong sexual selection.
392

393 **MATERIALS AND METHODS**

394 **Worm culture and mating design**

395 We previously evolved six replicated populations of *C. elegans* pseudo-females (*fog-2*) and
396 males under enhanced post-insemination competition for 30 generations [20]. The complete
397 experimental evolution details for the between-strain post-insemination selection only (BS-PO)
398 regime can be found in Kasimatis et al. (2022) [20]. Briefly, evolving males mated with females
399 for 24 hours after which male sterility was induced to prevent further sperm transfer. Fully fertile
400 competitor males were then added to the population to generation sperm competition. After a 24-
401 hour sperm competition period, progeny coming from the evolving males only were collected
402 and propagated to the next generation. Hence, selection was acting on sperm defensive capability
403 and sperm longevity. Here we chose four evolved experimental evolution replicates (A2, A3, B2,
404 B3), which spanned the range of reproductive increase, for transcriptomic analysis.
405 To generate mated male transcriptomes, we crossed ancestral (strain PX632) females to
406 ancestral (strain PX632) males and evolved (G31 replicates A2, A3, B2, B3) females to evolved
407 (G31 replicates A2, A3, B2, B3) males. The evolved crosses were performed for each
408 experimental evolution replicate. To start a generation, age synchronized larval stage 1 (L1)
409 worms were plated onto a 10 cm NGM-agar plates seeded with OP50 *Escherichia coli* at 20ºC
410 with a density of 1,000 worms per plate [47,48]. Late larval stage 4 (L4) females (n = 40) and
411 males (n = 40) were isolated onto medium NGM-agar plates (60 mm diameter) seeded with
412 100µL OP50 *E. coli*. Three mating plates were set up for each cross. Mating plates were kept at
413 20ºC for 48 hours. After the 48-hour mating period, males were collected and pooled (n = 120)

414 from all three mating plates per cross for bulk RNA isolation. Three biological replicate mating
415 assays were conducted for each cross, each coming from an independent age synchronization
416 event (n = 15 total male samples).
417 To generate mated female transcriptomes, we used a factorial design of ancestral (strain
418 PX632) and evolved (G31 replicates A2, A3, B2, B3) females mated to ancestral (strain PX632)
419 and evolved (G31 replicates A2, A3, B2, B3) males. Crosses were performed for each
420 experimental evolution replicate following the same experimental procedure as males. After the
421 48-hour mating period, females were collected and pooled (n = 120) from all three mating plates
422 per cross for bulk RNA isolation. Three biological replicate mating assays were conducted for
423 each cross, each coming from an independent age synchronization event (n = 39 total female
424 samples).

425 **Transcriptome sequencing, mapping, and gene calling**

426 We performed bulk mRNA sequencing of mated males and females, separately, on day 3 of
427 adulthood. Worms were isolated into 150uL of S-Basal buffer and then pelleted to ~20uL. They
428 were preserved with 250uL Tri-reagent and flash frozen in liquid nitrogen. The samples went
429 through 10 freeze-thaw cycle before RNA was isolated. RNA was isolated using the KAPA
430 mRNA HyperPrep Kit (KK8580) with Illumina compatible adapters. Libraries were prepared
431 using the TrueSeq RNA Library Prep kit (Illumina) starting from 100 ng of RNA. 100bp single
432 reads for males and 100bp paired-end reads were sequenced on an Illumina HiSeq 4000 at the
433 University of Oregon Genomics and Cell Characterization Core Facility (Eugene, OR).
434 Reads were trimmed using skewer v0.2.2 [49] to remove low quality bases and TrueSeq
435 adapters (parameters: -x AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -y
436 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT -t 12 -l 30 -r 0.01 -d 0.01 -q 10). The
437 trimmed reads were mapped to the *C. elegans* N2 reference genome (PRJNA13758-WS274) [29]
438 through two passes using STAR v2.5.3a [50]. A table of gene counts was made using HTSeq
439 v0.9.1 [51] (parameters: -s no -r pos -i ID -t gene -f) based on *C. elegans* N2 reference genome
440 "longest isoform" annotations.

441 **Differential gene expression analysis**

442 To compare-and-contrast changes in gene expression over time based on the generation (*i.e.,* G0
443 or G31) of individuals at the time of mating, we tested for differential gene expression using the

444 package DESeq2 v1.34.0 [52] in R [53]. DESeq2 uses the generalized linear model framework to
445 estimate the degree to which genes are differentially expressed. We first analyzed each
446 experimental evolution replicate independently. For males, we fit the model: gene counts ~
447 $male_{Anc,Evo}$. Since we had a full factorial design for mated females, we fit the model: gene counts
448 ~ $female_{Anc,Evo}$ + $male_{Anc,Evo}$. In females, experimental evolution replicate B3 had two outlier
449 mating replicates (20_S36_L005 and 21_S37_L005), which were subsequently censored from
450 the dataset (Fig S5). We then combined all experimental evolution replicates to capture only the
451 expression changes that were repeatable across experimental evolution and reanalyzed the
452 differential gene expression model. Normalized count data and gene expression fold change
453 values were extracted from the DESeq2 linear models. However, significance values were
454 determined by fitting a generalized linear model in R with generation and replicate as fixed
455 effects. This approach is more robust to variation between experimental evolution replicates and
456 therefore provides a more conservative estimate significantly differentially expressed genes.
457 Significance was based on a Bonferroni cut-off. These censored, combined data were used for
458 subsequent analyses (Files S1).
459 We crossed referenced the genes with significant differential expression with the
460 genomic significance peaks identified in Kasimatis et al. (2022) [20]. Additionally, we examined
461 whether the *cis*-regulatory regions of significant differentially expressed genes – defined as 2kb
462 upstream of the start codon – had any SNPs with a significant change in allele frequency, but
463 none of these met the significance peak threshold defined in Kasimatis et al. (2022) [20] (File
464 S2).
465 We tested for enrichment of gene ontology terms and KEGG (Kyoto Encyclopedia of
466 Genes and Genomes) pathways in significantly differentially expressed genes using the package
467 goseq [54] in R [53]. goseq performs gene ontology analysis taking transcript length biases into
468 account. Median transcript lengths were calculated using from
469 TxDb.Celegans.UCSC.ce11.ensGene [55] based on the UCSC Genome Browser *C. elegans*
470 genome version "ce11". Gene ontology enrichment was calculated using the "Wallenius" method
471 with a 0.05 false-discovery rate cutoff [56] (File S3).

472 **Sex-biased gene expression and dosage compensation**

473 We compared the change in expression of significant genes with their wildtype expression
474 profiles in *C. elegans fog-2* females and males [21] to examine any potential changes in sex-

475 biased expression profiles. The relationship between sex-biased expression and differentiation
476 gene expression in each sex was analyzed by fitting a linear model in R.
477 The dosage compensation profiles for *C. elegans* hermaphrodites (XX individuals) and
478 males (XO individuals) were taken from Jans et al. (2009), as were the positions of *rex* and *dox*
479 binding sites. We then correlated dosage compensation profiles with all genes in our
480 transcriptomic dataset. The positions of *rex* and *dox* sites were compared with genomic SNP data
481 from Kasimatis et al. (2022) [20] to determine if any significant allele frequency changes
482 occurred in these regions.
483
484 **Data Accessibility:** Raw transcriptomic reads are available at NCBI SRA under accession
485 number PRJNA1013082. Differential expression analysis results for the combined data are in
486 File S1. Cross-referencing of *cis*-regulatory SNPs are available in File S2. Gene ontology and
487 KEGG pathway enrichment results are available in File S3. All scripts are publicly available on
488 the GitHub repository: https://github.com/katjakasimatis/expevol_femaletranscriptomics. The
489 experimental evolution ancestral strain (PX632) and evolved replicates are available from the
490 Phillips lab upon request.
491
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497
498 **Author Contributions:** KRK and PCP devised the project. KRK and CS performed the crosses.
499 JHW prepared the transcriptomic libraries. KRK analyzed the data. KRK wrote the manuscript
500 with the support of the other authors.
501

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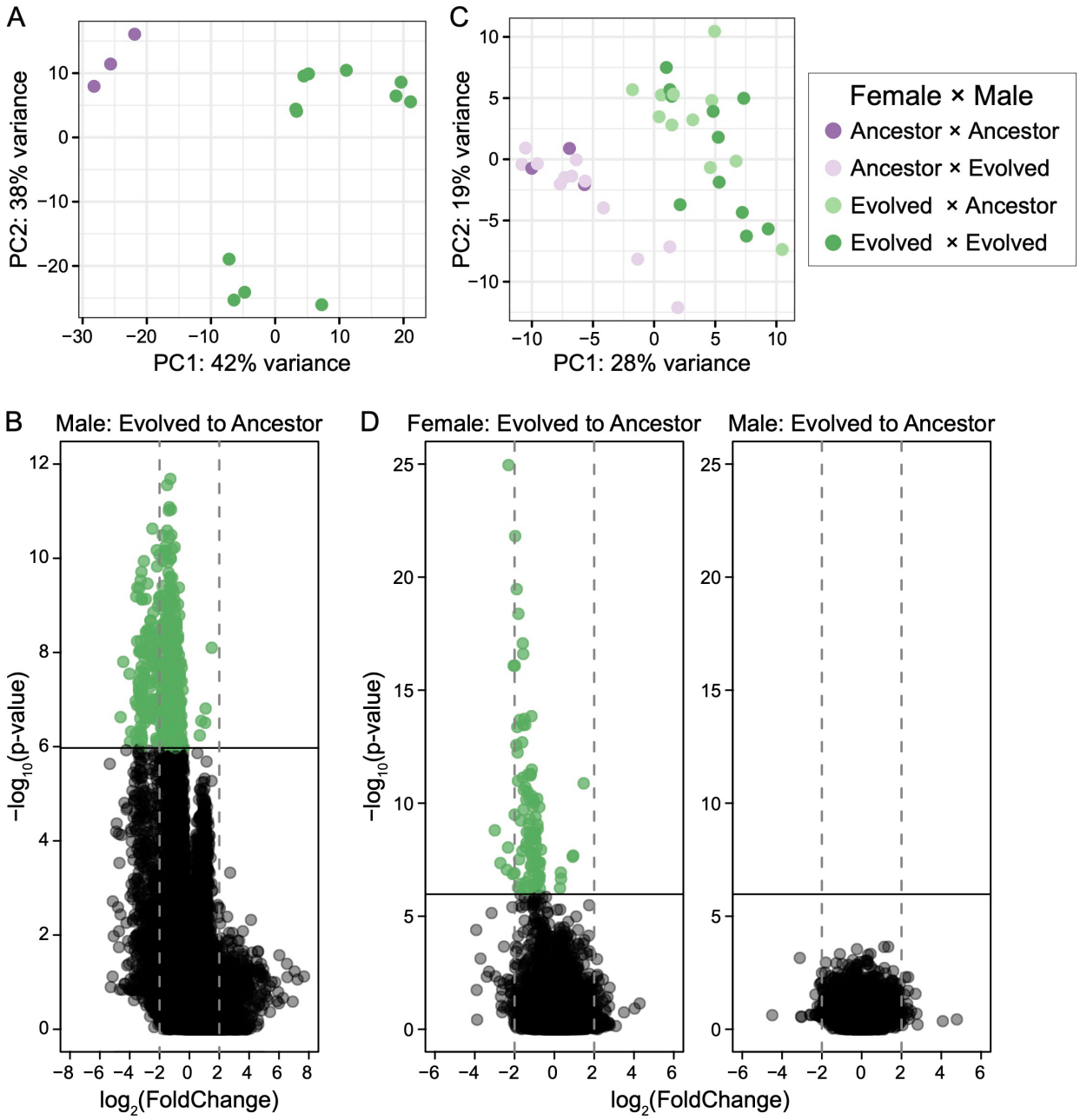

Figure 1. Differential gene expression is partitioned by ancestral (purple) and evolved (green) individuals. **A)** Principal component analysis of the variance stabilized dataset partitions male generation along PC1. **B)** Differential gene expression was analyzed based on male generation and experimental evolution replicate. Only the male term contributed to gene expression changes (File S1). Genes with a significant change in expression from ancestral to evolved males (shown in green) were determined using a Bonferroni cut-off. Positive expression changes correspond to upregulation in evolved males, while negative expression changes correspond to downregulation

621 in evolved males. **C)** Principal component analysis of the variance stabilized dataset partitions
622 female generation along PC1. **D)** Differential gene expression was analyzed based on female
623 generation, male generation, and experimental evolution replicate. Only the female term
624 contributed to gene expression changes. Genes with a significant change in expression from
625 ancestral to evolved females (shown in green) were determined using a Bonferroni cut-off.
626 Positive expression changes correspond to upregulation in evolved females, while negative
627 expression changes correspond to downregulation in evolved females.
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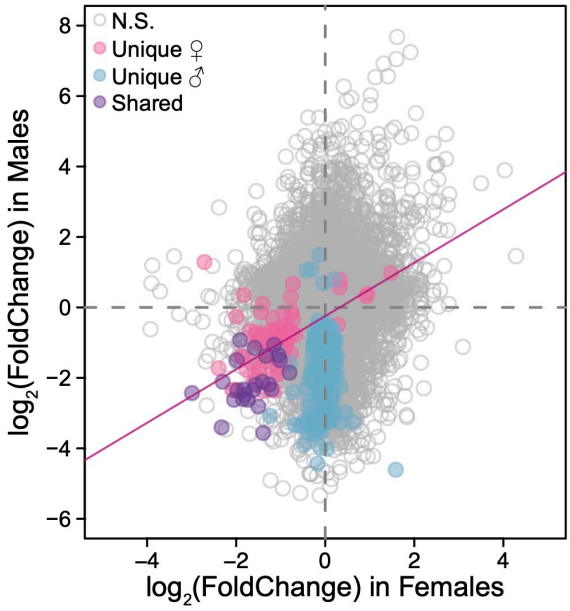

Figure 2. A comparison of changes in gene expression levels between females and males. Positive expression changes correspond to upregulation in evolved females/males, while negative expression changes correspond to downregulation in evolved females/males. Genes that are not significantly differentially expressed in either sex are shown in gray. Genes that are significantly differentially expressed in males but not in females (*i.e.*, unique male) are shown in blue. Genes that are significantly differentially expressed in females but not in males (*i.e.*, unique female) are shown in pink. Genes that are significantly differentially expressed in both sexes (*i.e.*, shared) are shown in purple. There is a significant relationship between the change in gene expression in females and males for all female DEs ($F_{1,98} = 42.16$, $p < 0.001$, adjusted $R^2 = 0.29$).

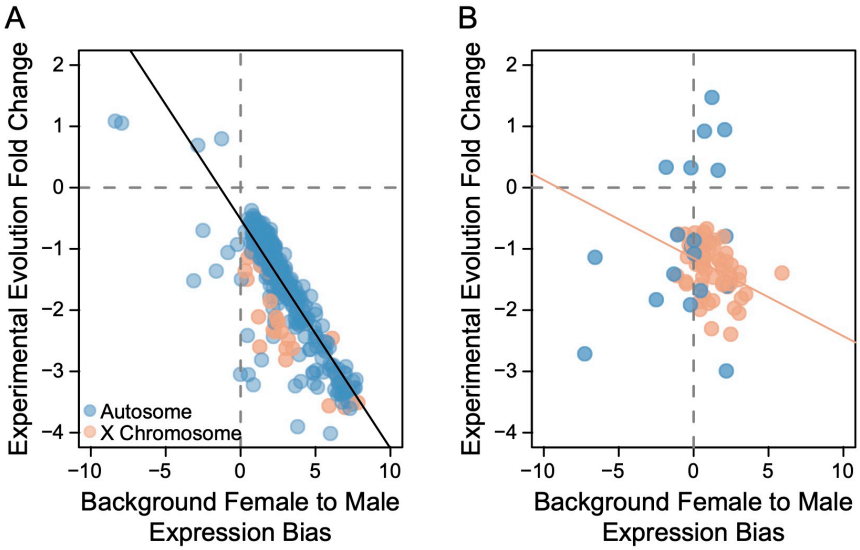

Figure 3. Downregulated genes are female-biased. **A)** There is a significant negative correlation between wildtype sex-bias in expression and changes in expression after experimental evolution in males. This relationship holds regardless of genomic location. Positive wildtype gene expression values indicate female-biased expression and negative values indicate male-biased expression. Both sex-biased expression (female to male) and experimental evolution fold change are plotted on a $\log_2$ scale. **B)** There is a negative correlation between significant differentially expressed genes located on the X chromosome (shown in coral) and sex-biased expression in females. Only 22 genes were located on autosomes (shown in blue).

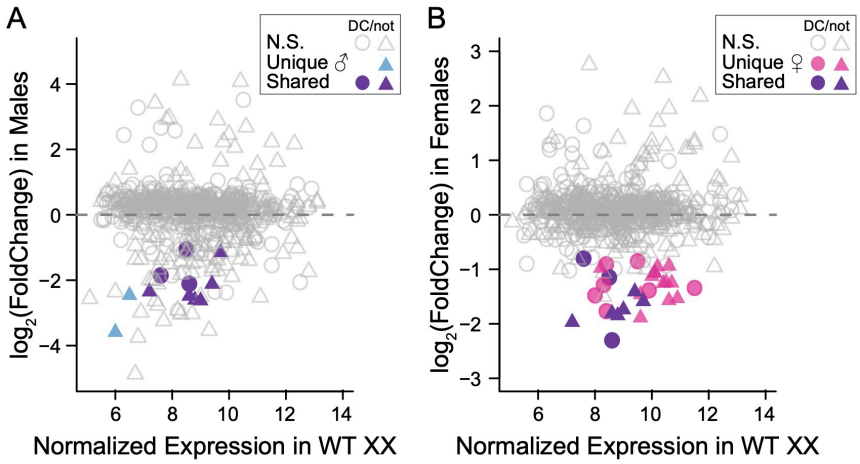

Figure 4. Downregulation of X-linked genes is impacting dosage compensation. **A)** The relationship between normalized expression of dosage compensated (circles) and not dosage compensated (triangles) X-linked genes in wildtype hermaphrodites/females (XX individuals) versus differential gene expression in males. Genes with significant differential gene expression in males only are shown in blue and shared genes are shown in purple. Both axes are plotted on a $\log_2$ scale. **B)** The relationship between normalized expression of dosage compensated (circles) and not dosage compensated (triangles) X-linked genes in wildtype hermaphrodites/females (XX individuals) versus differential gene expression in females. Genes with significant differential gene expression in females only are shown in pink and shared genes are shown in purple.

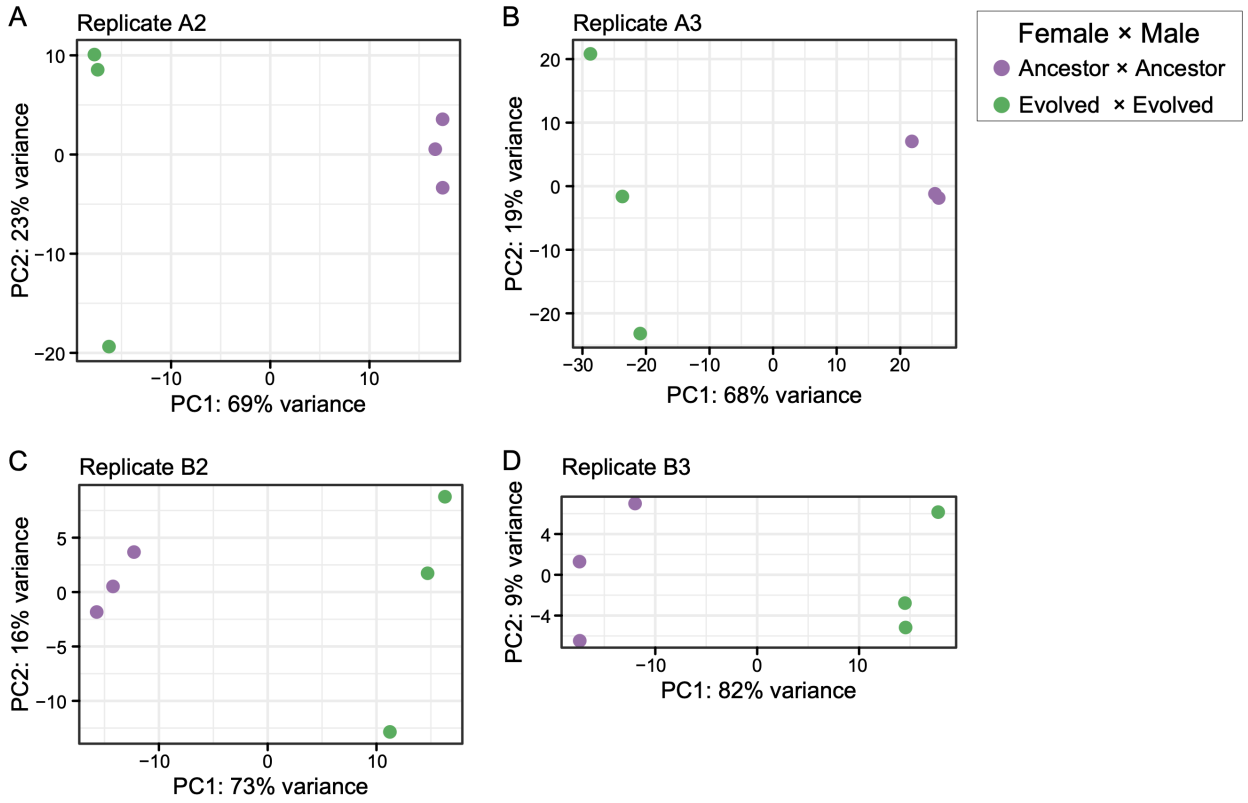

Figure S1. Principal component analysis of the variance stabilized data for each experimental evolution replicate partitions male generation along PC1.

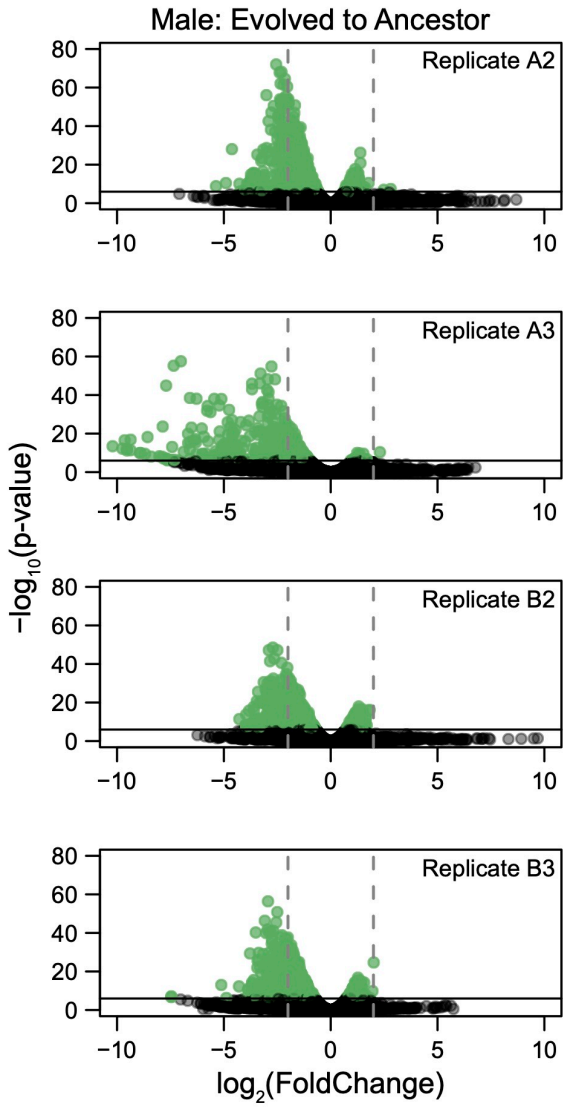

Figure S2. Differential gene expression in males for each experimental evolution replicate was analyzed based on male generation. Genes with a significant change in expression from ancestral to evolved males (shown in green) were determined using a Bonferroni cut-off. Positive expression changes correspond to upregulation in evolved males, while negative expression changes correspond to downregulation in evolved males.

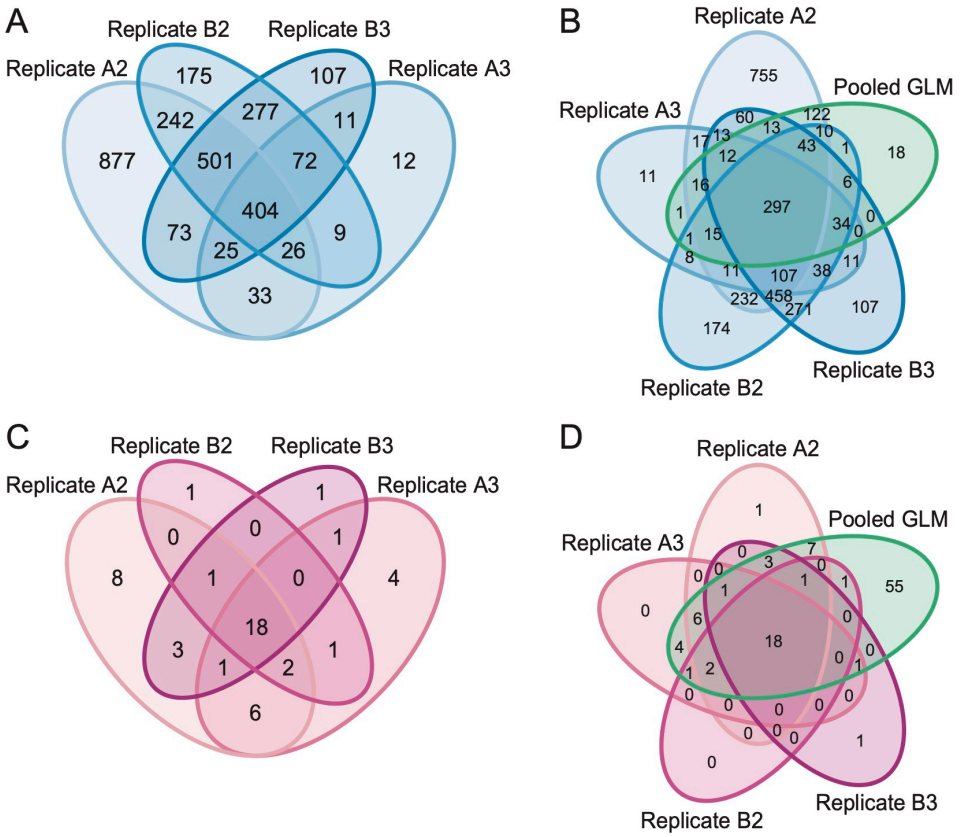

Figure S3. The overlap of significant genes across individual experimental evolution replicates in: **A)** males and **C)** females. The overlap between the individual experimental evolution replicate analyses and the combined analysis in: **B)** males and **D)** females.

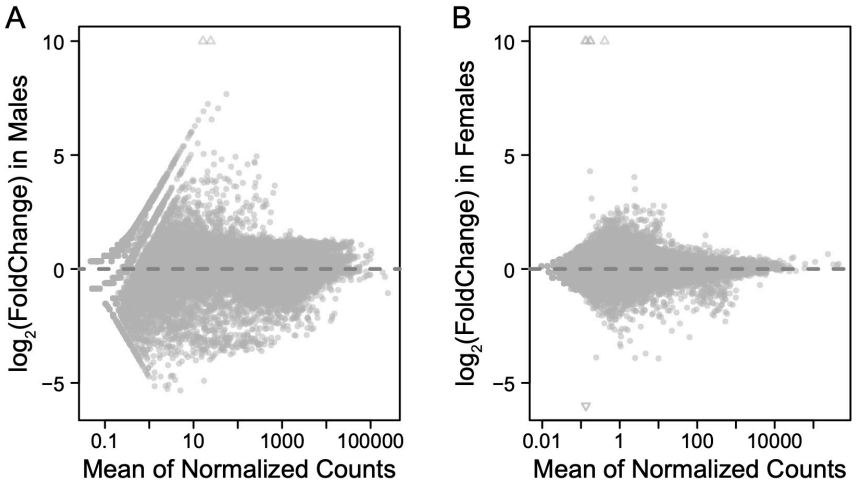

Figure S4. Distribution of normalized gene expression counts against the change in gene expression for the combined analysis of: **A)** males and **B)** females. Triangles represent points that fall outside of the y-axis range.

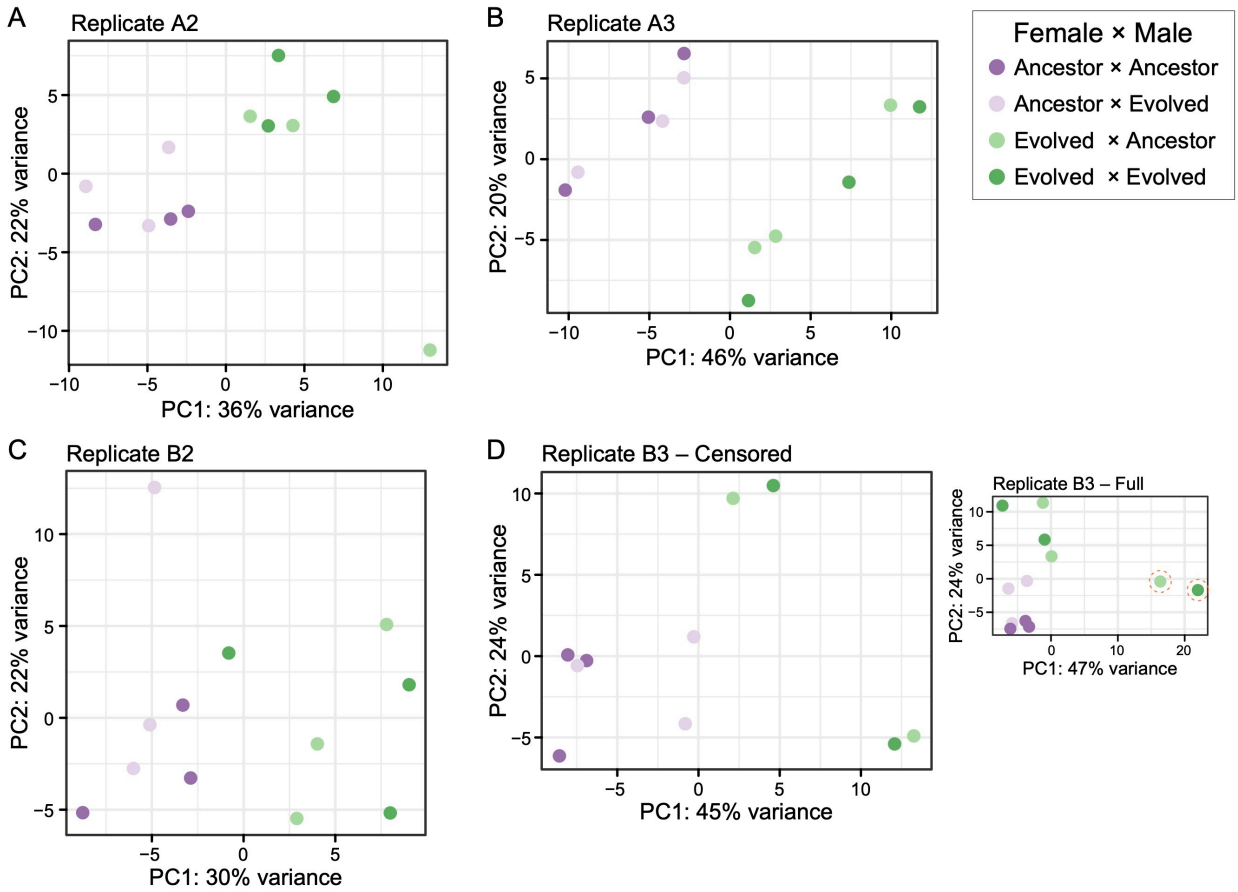

Figure S5. Principal component analysis of the variance stabilized data for each experimental evolution replicate partitions female generation along PC1. Experimental evolution replicate B3 had two outlier transcriptome replicates (circled) and were censored from the final, combined dataset.

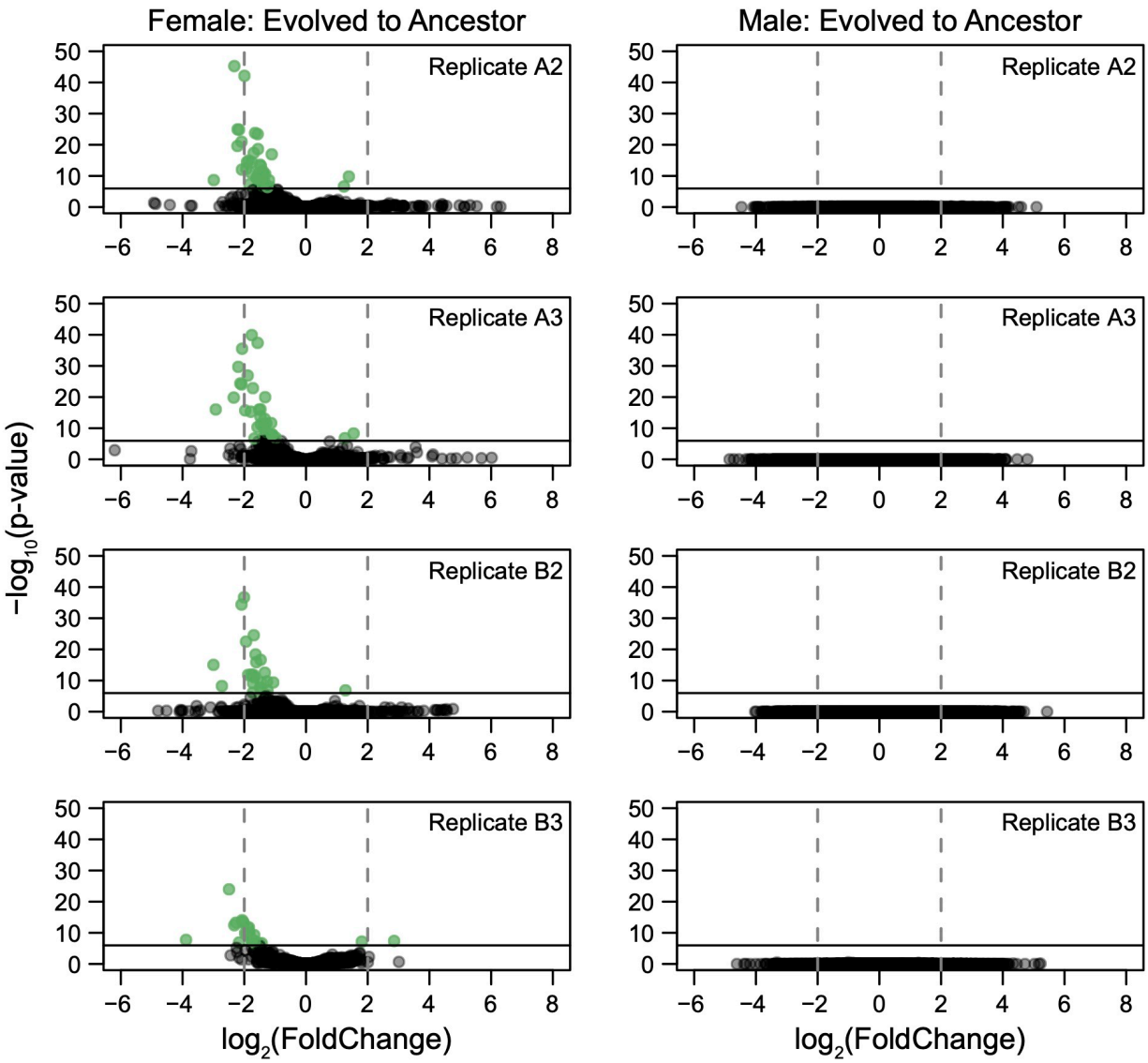

Figure S6. Differential gene expression in females for each experimental evolution replicate was analyzed based on female generation and male generation. Genes with a significant change in expression from ancestral to evolved females (shown in green) were determined using a Bonferroni cut-off. Positive expression changes correspond to upregulation in evolved females, while negative expression changes correspond to downregulation in evolved females.

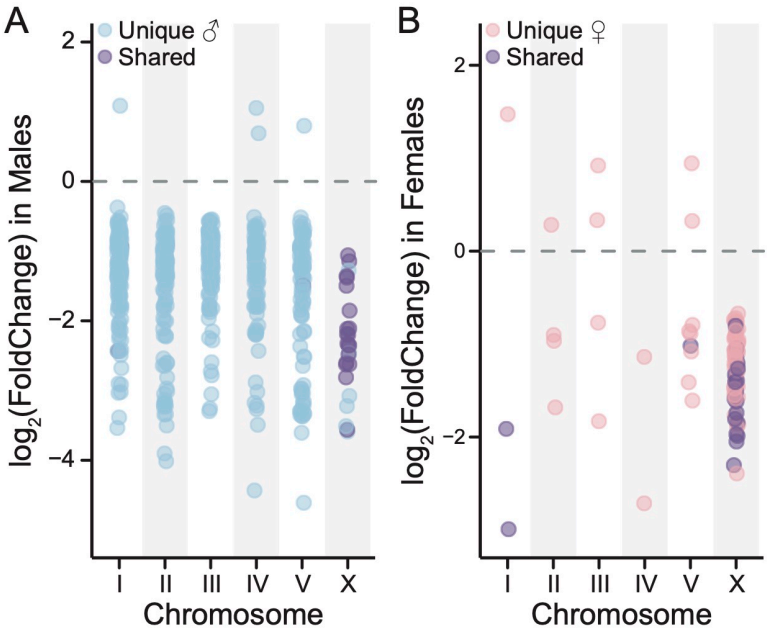

Figure S7. Chromosomal location of significantly differentially expressed genes. **A)** In males, the majority of DEs are located on the autosomes. Unique male DEs are shown in blue and shared DEs are shown in purple. **B)** In females, the majority of DEs are located on the X chromosome. Unique female DEs are shown in pink and shared DEs are shown in purple.

701 **File S1.** Differential expression analysis for the combined male and combined female datasets.

702

703 **File S2.** Genomic SNPs located in the cis-regulatory region of DEs.

704

705 **File S3.** Gene ontology analysis and KEGG Pathway analysis for DEs.