Post-insemination sexual selection in males indirectly masculinizes the female transcriptome

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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 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 is driven by female ancestral or evolved generation alone and that male generation has no impact on changes in gene expression. Almost all differentially expressed genes were downregulated in evolved females. Moreover, 80% of these gene were located on the X chromosome and have wildtype female-biased expression profiles. 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 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 sexual conflict in expression space.

LAY SUMMARY

Sexual selection drives the evolution of some of the most dramatic phenotypic differences between the sexes. Such sexual dimorphism is so common across multicellular organisms that we often overlook how remarkable it is for shared genetic material to create numerous and complex sex differences. At an evolutionary level, sexual dimorphism furthers the opportunity for sex-specific selection to optimize the fitness of a given sex. As a consequence, sex-specific selection, such as sexual selection, can have an indirect evolutionary response in the other sex due to genetic associations created by the sexes sharing the same genome. This correlated evolutionary response can create sexual conflict by shifting a sex away from their fitness optimum. At the functional level, sexual dimorphism is generated is through sex-specific regulation of gene expression. Bridging the evolutionary response to sexual selection with the evolution of sex-specific gene regulation during post-mating interactions has proved challenging. We previously used experimental evolution to increase male fertility by directly selecting for increased sperm competitive ability. In this study, we examined the effect of this direct selection on males on gene expression patterns in females. Differential gene expression was determined by whether a female was ancestral or evolved generation, indicating that gene expression changes were an evolved response due to indirect selection on females. Significantly differentially expressed genes were downregulated in evolved females. These genes tended to be female-biased in wildtype individuals and located on the X chromosome. The downregulation of X-linked genes suggests expression levels in females equal to or lower than that in males. Together these results indicate a less female-like transcriptome after experimental evolution. This supports a sexual conflict scenario by which direct sexual selection on males indirectly masculinizes the female transcriptome over short evolutionary timescales.

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 (Wyman *et al.*, 2013; Kasimatis *et al.*, 2017). On a functional level, this means that gene expression must be tightly regulated in a sex-specific manner (Grath & Parsch, 2016; Mank, 2017). 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 (Lande & Arnold, 1983, 1985). 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 (Fisher, 1930; Lande, 1981; Kirkpatrick, 1982). 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 (Turelli, 1985). 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 (Darwin, 1871; Cooper & Forshaw, 1977). 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 (Lande, 1980). The degree to which mating traits have additive genetic covariance, then, determines the opportunity for sexual conflict (Gavrilets *et al.*, 2001; Arnqvist & Rowe, 2005). 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

101 interacts with the male ejaculate to affect female fertilization success. Sexual selection on male
102 reproductive proteins has been inferred through molecular evolution analyses (Begun *et al.*,
103 2000; Swanson & Vacquier, 2002; Clark *et al.*, 2006; Chapman, 2011). These studies indicate
104 that many seminal fluid proteins evolve rapidly due to sexually antagonistic coevolution, which
105 in turn implies that direct selection is acting on both female and male post-insemination traits.
106 However, identifying the specific female traits with which these male reproductive proteins
107 interact has proved challenging. In this case, transcriptomic analysis can be useful for identifying
108 wide-spread changes in gene expression after mating to uncover polygenic responses to selection
109 within and across generations.

110 Few studies have measured direct selection on male post-insemination traits and the
111 correlated response in females in a similar manner to male display and female preference traits.
112 We previously used experimental evolution to select for increased sperm competitive ability in
113 *C. elegans* males (Kasimatis *et al.*, 2022). Experimental evolution provides an opportunity to
114 reproducibly isolate directional selection on male characteristics from the potentially correlated
115 responses in females using timescales when the opportunity for sexual conflict is likely to be
116 highest. We found that after 30 generations of evolution, males showed a strong, rapid response
117 to post-insemination selection at both the phenotypic and genomic levels (Kasimatis *et al.*,
118 2022). Specifically, post-insemination reproductive success increased by greater than four-fold in
119 the evolved populations. This reproductive fitness increase was underlain by a polygenic
120 response of 82 significance peaks represented by 57 genes, 10 intergenic regions, and 15 putative
121 pseudogenes. Here we test if this strong, direct selection acting on male sperm competitive
122 ability had an indirect impact on females. Specifically, we examine the transcriptomes of mated
123 females using a fully factorial crossing design of ancestral and evolved individuals to determine:
124 i) whether strong, direct selection on sperm competition generates a response in females, and if
125 so, ii) is the female response evolved or plastic, and iii) is the female response indicative of
126 sexual conflict.
127

128 **MATERIALS AND METHODS**

129 **Worm culture and mating design**

130 We previously evolved six replicated populations of *C. elegans* pseudo-females (*fog-2*) and
131 males under enhanced post-insemination competition for 30 generations (Kasimatis *et al.*, 2022).

132 The complete experimental evolution details for the between-strain post-insemination selection
133 only (BS-PO) regime can be found in Kasimatis *et al.* (2022). Briefly, evolving males mated
134 with females for 24 hours after which male sterility was induced to prevent further sperm
135 transfer. Fully fertile competitor males were then added to the population to generate sperm
136 competition. After a 24 hour sperm competition period, progeny coming from the evolving males
137 only were collected and propagated to the next generation. Hence, selection was acting on sperm
138 defensive capability and sperm longevity. Here we chose four evolved experimental evolution
139 replicates (A2, A3, B2, B3), which spanned the range of reproductive increase, for female
140 transcriptomic analysis.

141 For each experimental evolution replicate, we used a factorial design of ancestral (strain
142 PX632) and evolved (G31 replicates A2, A3, B2, B3) females mated to ancestral (strain PX632)
143 and evolved (G31 replicates A2, A3, B2, B3) males. To start a generation, age synchronized
144 larval stage 1 (L1) worms were plated onto a 10 cm NGM-agar plates seeded with OP50
145 *Escherichia coli* at 20ºC with a density of 1,000 worms per plate (Brenner, 1974; Kenyon *et al.*,
146 1993). Late larval stage 4 (L4) females (n = 40) and males (n = 40) were isolated onto medium
147 NGM-agar plates (60 mm diameter) seeded with 100µL OP50 *E. coli*. Three mating plates were
148 set up for each cross. Mating plates were kept at 20ºC for 48 hours. After the 48 hour mating
149 period, females were collected and pooled (n = 120) from all three mating plates per cross for
150 bulk RNA isolation. Three biological replicate mating assays were conducted for each cross,
151 each coming from an independent age synchronization event (n = 39 total samples).

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

153 We performed bulk RNA sequencing of mated females on day 3 of adulthood. Worms were
154 isolated into 150uL of S-Basal buffer and then pelleted to ~20uL. They were preserved with
155 250uL Tri-reagent and flash frozen in liquid nitrogen. RNA was isolated using the Direct-zol
156 RNA Miniprep kit (Zymo). Libraries were prepared using the TrueSeq Stranded mRNA Library
157 Prep kit (Illumina) starting from 100 ng of RNA. 100bp paired-end reads were sequenced on an
158 Illumina HiSeq 4000 at the University of Oregon Genomics and Cell Characterization Core
159 Facility (Eugene, OR).

160 Reads were trimmed using skewer v0.2.2 (Jiang *et al.*, 2014) to remove low quality bases
161 and TrueSeq adapters (parameters: -x AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -
162 y AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT -t 12 -l 30 -r 0.01 -d 0.01 -q 10). The

163 trimmed reads were mapped to the *C. elegans* N2 reference genome (PRJNA13758-WS274)
164 (Harris *et al.*, 2019) through two passes using STAR v2.5.3a (Dobin *et al.*, 2013). A table of
165 gene counts was made using HTSeq v0.9.1 (Putri *et al.*, 2022) (parameters: -s no -r pos -i ID -t
166 gene -f) based on *C. elegans* N2 reference genome "longest isoform" annotations.

167 **Differential gene expression analysis**

168 To compare-and-contrast changes in gene expression over time based on the generation (*i.e.,* G0
169 or G31) of individuals at the time of mating, we tested for differential gene expression using the
170 package DESeq2 v1.34.0 (Love *et al.*, 2014) in R (R Core Team, 2020). DESeq2 uses the
171 generalized linear model framework to estimate the degree to which genes are differentially
172 expressed. We fit the model: gene counts ~ $\text{female}_{\text{Anc,Evo}}$ + $\text{male}_{\text{Anc,Evo}}$ + $\text{female}_{\text{Anc,Evo}}$:$\text{male}_{\text{Anc,Evo}}$.
173 We first analyzed each experimental evolution replicate independently. The male (ancestor
174 versus evolved) and interaction ($\text{ancestor}_{\text{F,M}}$ versus $\text{evolved}_{\text{F,M}}$) contrasts for each experimental
175 evolution replicate both showed no gene expression changes based on a Bonferroni cut-off
176 applied to adjusted p-values. The female (ancestor versus evolved) contrast had 51, 42, 23, and
177 32 significantly differentially expressed genes in experimental evolution replicates A2, A3, B2,
178 and B3, respectively (File S1). Principal component analysis of the variance stabilized data
179 supported variance partitioning by female, but not male, generation (Fig. S1). Experimental
180 evolution replicate B3 had two outlier mating replicates (20_S36_L005 and 21_S37_L005),
181 which were subsequently censored from the dataset. Given the consistency in results, we pooled
182 all experimental evolution replicates and reanalyzed the differential gene expression model. To
183 account for multiple testing, p-values were adjusted using a Benjamin and Hochberg correction
184 and significance was based on a Bonferroni cut-off. These censored, pooled data are represented
185 in the results (Files S1 and S2).

186 The molecular and biological functions of the genes with significant differential
187 expression were determined using gene ontology analysis in UniProt (Consortium, 2021) and
188 QuickGO (Huntley *et al.*, 2015). General reproduction keywords were taken from the WormBase
189 gene descriptions (Harris *et al.*, 2019). We tested for enrichment of gene ontology terms in
190 significantly differentially expressed genes using the package goseq (Young *et al.*, 2010) in R (R
191 Core Team, 2020). goseq performs gene ontology analysis taking transcript length biases into
192 account. Median transcript lengths were calculated using from
193 TxDb.Celegans.UCSC.ce11.ensGene (Team & Maintainer, 2019) based on the UCSC Genome

194 Browser *C. elegans* genome version "ce11". Gene ontology enrichment was calculated using the
195 "Wallenius" method with a 0.05 false-discovery rate cutoff (Benjamini & Hochberg, 1995) (File
196 S3).
197 We crossed referenced the genes with significant differential expression with the
198 genomic significance peaks identified in Kasimatis *et al.* (2022). Additionally, we examined
199 whether the *cis*-regulatory regions of significant differentially expressed genes – defined as 2kb
200 upstream of the start codon – had any SNPs with a significant change in allele frequency, but
201 none of these met the significance peak threshold defined in Kasimatis *et al.* (2022) (File S4).

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

203 We compared the change in expression of significant genes with their wildtype expression
204 profiles in *C. elegans fog-2* females and males (Albritton *et al.*, 2014) to examine any potential
205 changes in sex-biased expression profiles. To determine if significantly differentially expressed
206 genes were more female- or male-biased, we used a χ^2 test on the 1x3 contingency table of
207 significant genes with wildtype female-biased, non-biased, or male-biased expression. The
208 relationship between sex-biased expression and differentiation gene expression was analyzed by
209 fitting a linear model in R.
210 The dosage compensation profiles for *C. elegans* hermaphrodites (XX individuals) and
211 males (XO individuals) were taken from Jans et al. (2009), as were the positions of *rex* and *dox*
212 binding sites. We then correlated dosage compensation profiles with all genes in our
213 transcriptomic dataset. To determine if difference in dosage compensation profiles varied
214 between significant and non-significant differentially expressed genes, we used a χ^2 test on the
215 2x2 contingency table. The positions of *rex* and *dox* sites were compared with genomic SNP data
216 from Kasimatis *et al.* (2022) to determine if any significant allele frequency changes occurred in
217 these regions.
218

219 **RESULTS**

220 **A strong-inference framework for expression evolution**

221 We used a full factorial crossing design of ancestral and evolved females mated with ancestral
222 and evolved males from four experimental evolution replicates that were evolved under direct
223 selection on sperm competitive ability. Populations of individuals for each cross were mated for
224 48 hours after which females were isolated for transcriptomic analysis. The ancestor_F-ancestor_M

225 cross represents the baseline transcriptomic profile of females after mating before experimental
226 evolution occurred. The evolved$_F$-evolved$_M$ cross represents the transcriptomic profile of females
227 after 30 generations of evolution. If direct sexual selection on males had no impact on a gene's
228 expression level in females, then there would not be differential expression between the
229 ancestor$_F$-ancestor$_M$ and evolved$_F$-evolved$_M$ cross for that gene. Such genes would have no
230 genetic covariance between females and males.

231 If instead a gene is differentially expressed between the ancestor$_F$-ancestor$_M$ and
232 evolved$_F$-evolved$_M$ crosses, then females are responding to the sexual selection exerted on males.
233 If the female transcriptional changes are a plastic response to increased sperm competitiveness,
234 then male generation should be a major predictor, that is females of the evolved$_F$-ancestor$_M$ cross
235 should have expression profiles similar to the ancestor$_F$-ancestor$_M$ cross and females of the
236 ancestor$_F$-evolved$_M$ should have expression profiles similar to the evolved$_F$-evolved$_M$ cross.
237 Alternatively, if female transcriptional changes are a result of either direct selection in females
238 induce by sperm competitiveness or indirect selection generated by genetic covariation between
239 the sexes, then the expectation is that ancestral females will have similar expression profiles and
240 evolved females will have similar expression profiles, regardless of male generation.

241 We analyzed changes in gene expression for each experimental evolution replicate
242 separately (Fig S1). Since the same pattern was observed, we pooled all four experimental
243 evolution replicates, excluding the two outlier transcriptome replicates in experimental evolution
244 replicate B3, for greater power. The final censored, pooled dataset therefore consisted of three
245 independent ancestor$_F$-ancestor$_M$ crosses, 12 independent ancestor$_F$-evolved$_M$ crosses, 11
246 independent evolved$_F$-ancestor$_M$ crosses, and 11 independent evolved$_F$-evolved$_M$ crosses.

247 **Female generation determines gene expression changes**

248 Principal component analysis (PCA) was performed to compare samples based on female and
249 male generation (*i.e.,* G0 or G31) during mating. The first principal component explained 28% of
250 the total variance in gene expression levels based on female generation alone, such that all
251 ancestral females clustered together and all evolved females clustered together, regardless of
252 male generation (Fig 1A). Male identity did not contribute to variance partitioning in the first
253 two principal components, which captured 46% of the total variance.

254 Differential gene expression was analyzed using a model that fit female generation, male
255 generation, and the interaction between female and male generations. Neither male generation

256 nor the interaction term contributed to changes in gene expression (Fig 1B). Rather, female
257 generation determined differential gene expression, indicating an evolved expression response
258 (Fig 1B). Sixty-four genes showed significant differential gene expression of which 11 genes had
259 at least a two-fold change in expression (Fig 1B; Fig 2). Only three genes were significantly
260 upregulated in evolved females (mean fold increase = 1.2). All three genes are uncharacterized in
261 function and have no ontology terms relating them to reproduction (File S2). The other 61
262 significant genes were downregulated (mean fold decrease = 1.5) in evolved females relative to
263 ancestral females (Fig 1B; Fig 2). To determine if SNPs in the coding region or the *cis*-
264 regulatory region of these genes were responsible for the changes in expression, we examined
265 allele frequencies in our previously published genomic mapping data for these populations
266 (Kasimatis *et al.*, 2022). None of the significantly differentially expressed genes had significant
267 allele frequency changes in the coding region or the *cis*-regulatory region (File S2 & S4). Thus,
268 changes in gene expression were likely regulated through *trans* acting pathways.
269 To assess the functional pathways and biological processes in which these downregulated
270 genes were involved, we examined their associated gene ontology (GO) terms (File S1). The
271 most common molecular functions were DNA binding (n = 4), hydrolase activity (n = 4), and
272 transferase activity (n = 4). The most common biological process was vesicle-mediated transport
273 (n = 9) followed by membrane organization (n = 3) and lipid metabolic processes (n = 3). Six
274 genes were pseudogenes. Seventeen genes (28%) were uncharacterized in both molecular
275 function and biological process. Nineteen genes (31%) had a keyword relating them to
276 reproduction in their WormBase description (Harris *et al.*, 2019). GO enrichment analysis
277 identified 10 ontology terms that were significantly enriched in differentially expressed genes,
278 including intracellular membrane-bounded organelle ($n_{DE} = 30$, $n_{total} = 4792$, $p < 0.00001$),
279 membrane-bounded organelle ($n_{DE} = 30$, $n_{total} = 4952$, $p < 0.00001$), and vesicle-mediated
280 transport ($n_{DE} = 10$, $n_{total} = 470$, $p < 0.00001$) (File S3).

281 **X Chromosome Masculinization**

282 If widespread downregulation in evolved females was a result of direct selection in females, then
283 we hypothesize that selection would be countering the effects of increased sperm
284 competitiveness by either upregulating female-biased genes or downregulating male-biased
285 genes. We examined the relationship between changes in gene expression and sex-biased
286 expression in wildtype females and males (Albritton *et al.*, 2014). The majority (n = 46) of genes

287 with a significant expression change (excluding the four pseudogenes) showed some degree of
288 female-biased expression in wildtype individuals ($\chi^2 = 53.5$, d.f. = 2, $p < 0.0001$) (Fig 3A). Four
289 genes had male-biased expression, and only three were male-biased by at least an order of
290 magnitude difference. Interestingly, 48 of the downregulated genes (80%) were located on the X
291 chromosome, of which 40 were female-biased (Fig 3A). There is a negative relationship between
292 sex-biased expression and differential gene expression on the X chromosome, such that more
293 female-biased genes had a greater fold decrease in expression ($t = -2.71$, d.f. = 46, $p < 0.01$). This
294 relationship suggests a pleiotropic effect in a *trans*-regulatory pathway impacting regulation of
295 X-linked genes. No relationship exists between autosomal genes and differential gene expression
296 ($p = 0.26$), though the low number of autosomal genes likely makes this comparison under-
297 powered. Together, these data indicate a skew towards a less female-like transcriptome in
298 evolved females.
299 One major *trans*-regulatory pathway that could explain the correlation between
300 downregulated genes and the X chromosome is dosage compensation. In *Caenorhabditis*
301 nematodes, hermaphrodites/females have two copies of the X chromosome, each of which are
302 partially downregulated to match the dosage of the single X chromosome in males (Meyer,
303 2022). To determine if whole-scale changes in dosage compensation are responsible for the
304 downregulated pattern in evolved females, we correlated differential gene expression with gene
305 expression levels for dosage compensated and non-dosage compensated X-linked genes ($\chi^2 =$
306 4.97, d.f. = 1, $p = 0.03$) (Jans *et al.*, 2009). Only 23 (45%) of the significant genes located on the
307 X chromosome were present in both data sets (Fig. 3B). Of these, seven were dosage
308 compensated in wildtype hermaphrodites/females (XX individuals). These genes were further
309 downregulated in evolved females, potentially suggesting an expression level lower than males.
310 Sixteen genes were not dosage compensated in wildtype hermaphrodites/females, but were
311 downregulated in evolved females by on average 1.5-fold. Similarly, the mean fold decrease in
312 wildtype expression in males (XO individuals) for these same genes is 1.8-fold. The core dosage
313 compensation genes *sdc-2* ($\log_2$Fold Change = 0.02; $p_{adj} = 0.9$), *sdc-3* ($\log_2$Fold Change = -0.15;
314 $p_{adj} = 0.9$), *dpy-27* ($\log_2$Fold Change = -0.07; $p_{adj} = 1.0$), and *dpy-30* ($\log_2$Fold Change = 0.04;
315 $p_{adj} = 1.0$) did not have significant changes in gene expression after experimental evolution nor
316 were there significant allele frequency changes at or near these genes (Kasimatis *et al.*, 2022).
317 Additionally, there were no significant allele frequency changes at the *rex* or *dox* binding sites,

318 indicating that the dosage compensation machinery itself is likely not the cause of widespread
319 downregulation in evolved females. Therefore, alternative pathways must be involved in this
320 masculinization of the female transcriptome resulting from direct sexual selection acting on
321 males.
322

323 **DISCUSSION**

324 Sexual selection acts in a sex-specific manner yet can have an indirect evolutionary response in
325 the other sex due to genetic associations created by their sharing the same genome (apart from
326 heterogametic sex chromosomes when present). When sexual selection acts on one sex, the other
327 sex can be pulled away from their sex-specific optimum as a correlated response to selection,
328 generating conflict between the sexes. Here we explored whether direct sexual selection on male
329 sperm competitive ability (Kasimatis *et al.*, 2022) generated indirect selection in females by
330 quantifying the ancestral and evolved transcriptomes of females mated with different male
331 generations.

332 The factorial mating design used here allowed for changes in gene expression to be
333 categorized as a plastic response to increased sperm competitiveness in males or an evolved
334 response in the females themselves. If females were predisposed to increased sperm interactions,
335 then male generation should have been a major contributor to gene expression changes. Such a
336 scenario is analogous to a pre-existing bias model of sexual selection (Ryan *et al.*, 1990) and can
337 lead to direct selection on female preference/resistance (Gavrilets *et al.*, 2001; Arnqvist & Rowe,
338 2005). However, we found no evidence that male evolutionary history impacted differential gene
339 expression. Rather, all expression changes were determined based on whether a female was from
340 the ancestral or evolved populations, indicating that differential gene expression was a result of
341 indirect selection on females during experimental evolution. This scenario is analogous to
342 Fisherian sexual selection where direct selection on male traits can generate an indirect response
343 on female preference for those traits if an additive genetic covariance exists (Lande, 1981). For
344 our post-insemination model, selection was directed on sperm competitive ability. The correlated
345 female trait is not immediately clear based on GO analysis. However, membrane-bounded
346 organelle, vesicle-mediated transport, and lipid metabolism may be related to oogenesis, while
347 DNA binding may be related to transcriptional activity. Since selection on males generated a

348 polygenic response and the expression changes observed were likely due to *trans*-acting
349 pathways, it is possible that more than one female reproductive trait may be involved.
350 Interestingly, there was a complete lack of overlap between the genic changes identified
351 in Kasimatis *et al.* (2022) and the expression changes characterized here. One potential reason
352 for this discrepancy is that the genic changes were altering expression of upstream transcription
353 factors that are expressed earlier in development. Kasimatis *et al.* (2022) identified genomic
354 changes in the coding sequence of or nearby intergenic region of four transcription factors. Of
355 particular note is the transcription factor *sea-1*, which is involved in sex determination and
356 dosage compensation through hypoactivation of the X chromosome (Harris *et al.*, 2019).
357 Genomic analysis identified seven SNPs with a significant allele frequency change located
358 within a 30 base pair region of intron 2 (Kasimatis *et al.*, 2022). It is possible that this region
359 changes regulation of *sea-1* itself or alters splicing in a way that increase the hypoactivation
360 function of *sea-1* and thus is responsible for the widespread downregulation of X-linked genes.
361 Genes with significant expression changes between ancestral and evolved females were
362 almost exclusively downregulated. Moreover, most of these genes are female-biased in wildtype
363 individuals. These results suggest that the evolved female transcriptome moved away from a
364 female-like state, supporting indirect rather than direct selection on females. The majority of
365 downregulated genes were located on the X chromosome. Because males are the heterogametic
366 sex in *C. elegans*, alleles affecting gene expression on the X chromosome are directly exposed to
367 selection without any potential shielding of an alternative allele due to dominance, as would
368 occur in heterozygous females. Thus, selection can be more effective on X-bearing genes,
369 especially when there is sex-specific selection (Charlesworth *et al.*, 1987; Mank, 2009; Vicoso &
370 Charlesworth, 2009). The response we observe here is thus consistent with the dominance theory
371 of the rapid evolution of sex chromosomes and their role in speciation (Charlesworth *et al.*,
372 1987; Charlesworth, 1991).
373 Despite the preponderance of signal on the X chromosome, we found no evidence that
374 mutations in the core dosage compensation pathway were the cause of this widespread
375 downregulation. Downregulation of dosage compensated genes suggests potentially lower
376 expression in evolved females relative to evolved males. In contrast, downregulation of non-
377 dosage compensated genes moved the evolved female expression level closer to the wildtype
378 male expression level. These changes further support a less female-like and more male-like

379 transcriptome. Since expression in males was not quantified, we cannot definitively say that
380 these genes were not equally downregulated in both sexes to maintain the sex bias and dosage
381 ratios. However, under such a scenario male generation should have been a significant predictor
382 of gene expression changes, which was not observed. Overall, the results indicate an indirect
383 masculinization of the female transcriptome generated by direct selection on male fertilization
384 success. Since gene expression levels impact trait function and, in turn, fitness, short-term sexual
385 conflict was likely generated as a byproduct of direct sexual selection in males as female
386 expression was pushed off its global optimum.

387 Previous experimental evolution studies suggest that male-limited evolution over tens of
388 generations can decrease female fitness (Rice, 1996; Prasad *et al.*, 2007; Pischedda &
389 Chippindale, 2017). Conversely, female-limited or reduced sexual selection evolution tends to
390 increase female fitness with little to no effect on male fitness (Holland & Rice, 1999; Pitnick *et*
391 *al.*, 2001a, 2001b). These previous studies therefore suggest that sex-specific selection acting
392 through males may have a higher potential to generate sexual conflict. However, this previous
393 work has been blind to the traits under selection and therefore the genetic architecture underlying
394 the sexual conflict response. Our work to bridge these layers of the genomic, transcriptomic, and
395 fitness responses suggests a Red Queen scenario (Valen, 1973; Brockhurst *et al.*, 2014) relating
396 genome evolution and transcriptional dimorphism during post-insemination interactions.
397 Specifically, on microevolutionary timescales strong sexual selection in males pushes females
398 away from their fitness optimum through indirect selection (Rice & Holland, 1997; Rice &
399 Chippindale, 2001). Females may then respond through direct selection, which creates a
400 macroevolutionary picture of a stable female-like state. Thus, examining the functional layers
401 through which sexual dimorphism arises is critical for understanding the context in which sexual
402 conflict can occur.

403 Sexual selection drives the evolution of some of the most elaborate phenotypic variation
404 and complex behaviors. We show that sexual selection is just as powerful an evolutionary force
405 on post-insemination traits. In particular, on microevolution timescales, females may not have
406 the opportunity to counter the effects of indirect selection leading to females being pulled away
407 from their optimal state. Given the seemingly high degree of pleiotropy between female and
408 male reproductive networks, sexual conflict may evolve very rapidly in populations experiencing
409 strong sexual selection.

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Data Accessibility: Raw transcriptomic reads will be made available at NCBI prior to publication. Differential expression analysis results for all data are in File S1. Analysis of significant genes are in File S2. Gene ontology enrichment results are available in File S3. Cross-referencing of *cis*-regulatory SNPs are available in File S4. All scripts are publicly available on the GitHub repository: https://github.com/katjakasimatis/expevol_femaletranscriptomics. The experimental evolution ancestral strain (PX632) and evolved replicates are available from the Phillips lab upon request.

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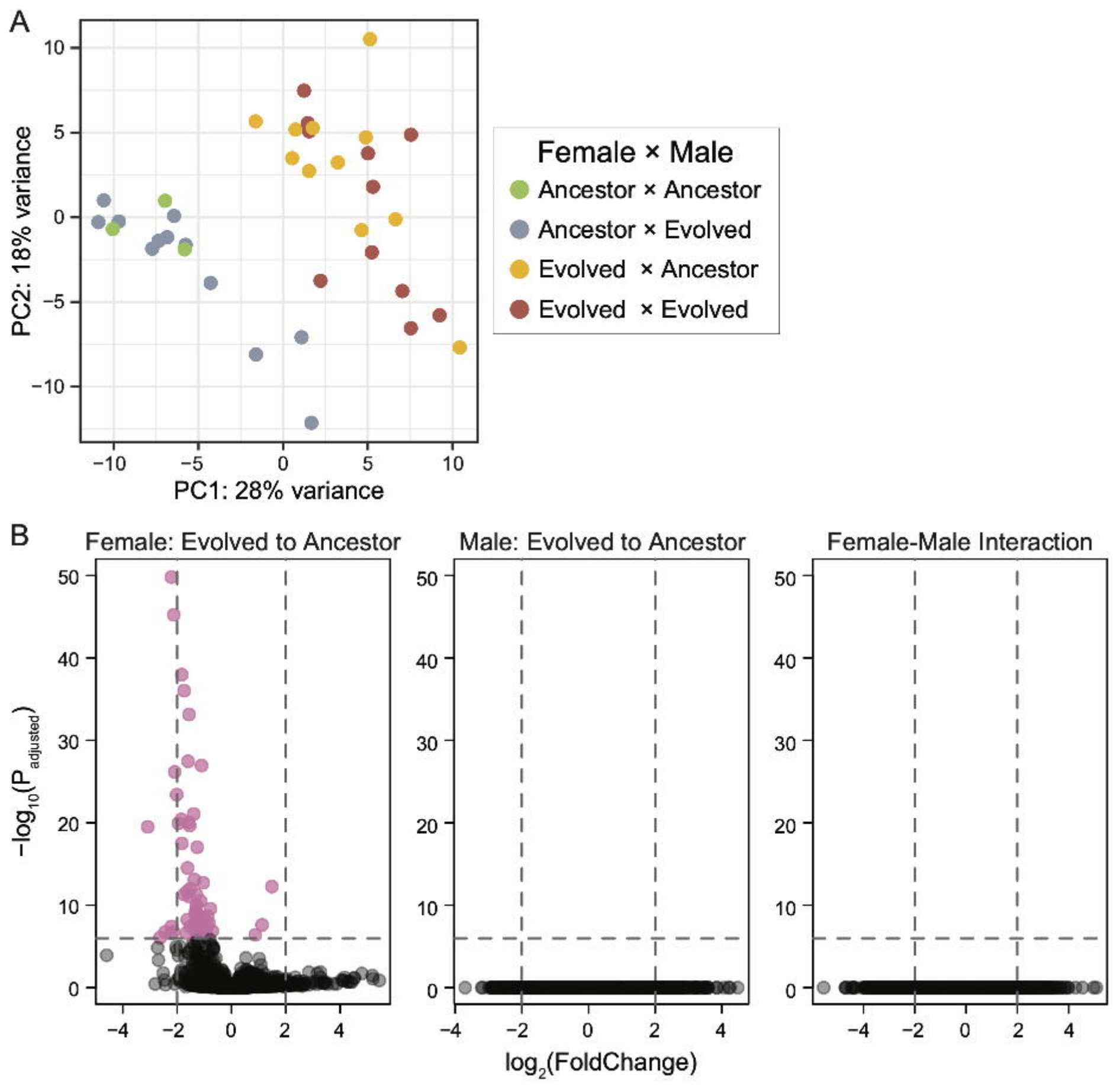
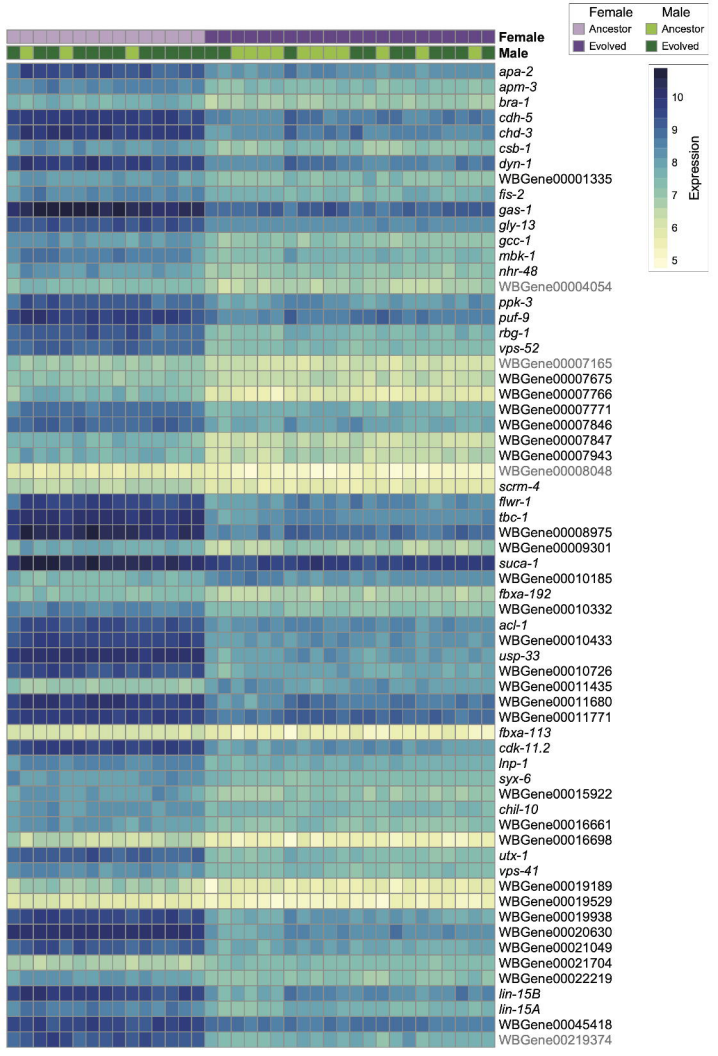
537 **Figure 1.** Differential gene expression is explained by female generation. **A)** Principal
538 component analysis of the variance stabilized dataset partitions female generation along PC1. **B)**
539 Differential gene expression was analyzed based on female generation, male generation, and the
540 interaction between female and male generations. Only the female contrast contributed to gene
541 expression changes. Genes with a significant change in expression from ancestral to evolved
542 females (shown in pink) were determined using a Bonferroni cut-off. Positive expression
543 changes correspond to upregulation in evolved females, while negative expression changes
544 correspond to downregulation in evolved females.
545
546 **Figure 2.** A heatmap of gene expression levels in significant differentially expressed genes.
547 Ancestral females (light purple) and evolved females (dark purple) group together, while
548 ancestral (light green) and evolved (dark green) males are interspersed. Sixty-one of the 64
549 significant genes are downregulated in evolved females. Pseudogenes are labeled in gray.
550
551 **Figure 3.** Downregulated genes are female-biased. **A)** There is a negative correlation between
552 significant differentially expressed genes located on the X chromosome (shown in coral) and
553 sex-biased expression. Only 13 genes were located on autosomes (shown in blue). Both sex-
554 biased expression (female to male) and experimental evolution fold change are plotted on a $\log_2$
555 scale. **B)** The relationship between normalized expression of dosage compensated (circles) and
556 not dosage compensated (triangles) X-linked genes in wildtype hermaphrodites/females (XX
557 individuals) versus differential gene expression. Genes with significant differential gene
558 expression are shown in pink. The dashed line represents the mean fold decrease of non-dosage
559 compensated genes in wildtype males (XO individuals). Both axes are plotted on a $\log_2$ scale.
560
561 **Figure S1.** Principal component analysis of the variance stabilized data for each experimental
562 evolution replicate partitions female generation along PC1. Experimental evolution replicate B3
563 had two outlier transcriptome replicates (circled) and were censored from the final, pooled
564 dataset.


A
PC2: 18% variance
PC1: 28% variance
Female × Male
Ancestor × Ancestor
Ancestor × Evolved
Evolved × Ancestor
Evolved × Evolved
B
Female: Evolved to Ancestor
Male: Evolved to Ancestor
Female-Male Interaction
$-\log_{10}(P_{adjusted})$
$\log_2$(FoldChange)


Female
Ancestor
Evolved
Male
Ancestor
Evolved
Female
Male
Expression
10
9
8
7
6
5
apa-2
apm-3
bra-1
cdh-5
chd-3
csb-1
dyn-1
WBGene00001335
fis-2
gas-1
gly-13
gcc-1
mbk-1
nhr-48
WBGene00004054
ppk-3
puf-9
rbg-1
vps-52
WBGene00007165
WBGene00007675
WBGene00007766
WBGene00007771
WBGene00007846
WBGene00007847
WBGene00007943
WBGene00008048
scrm-4
flwr-1
tbc-1
WBGene00008975
WBGene00009301
suca-1
WBGene00010185
fbxa-192
WBGene00010332
acl-1
WBGene00010433
usp-33
WBGene00010726
WBGene00011435
WBGene00011680
WBGene00011771
fbxa-113
cdk-11.2
lnp-1
syx-6
WBGene00015922
chil-10
WBGene00016661
WBGene00016698
utx-1
vps-41
WBGene00019189
WBGene00019529
WBGene00019938
WBGene00020630
WBGene00021049
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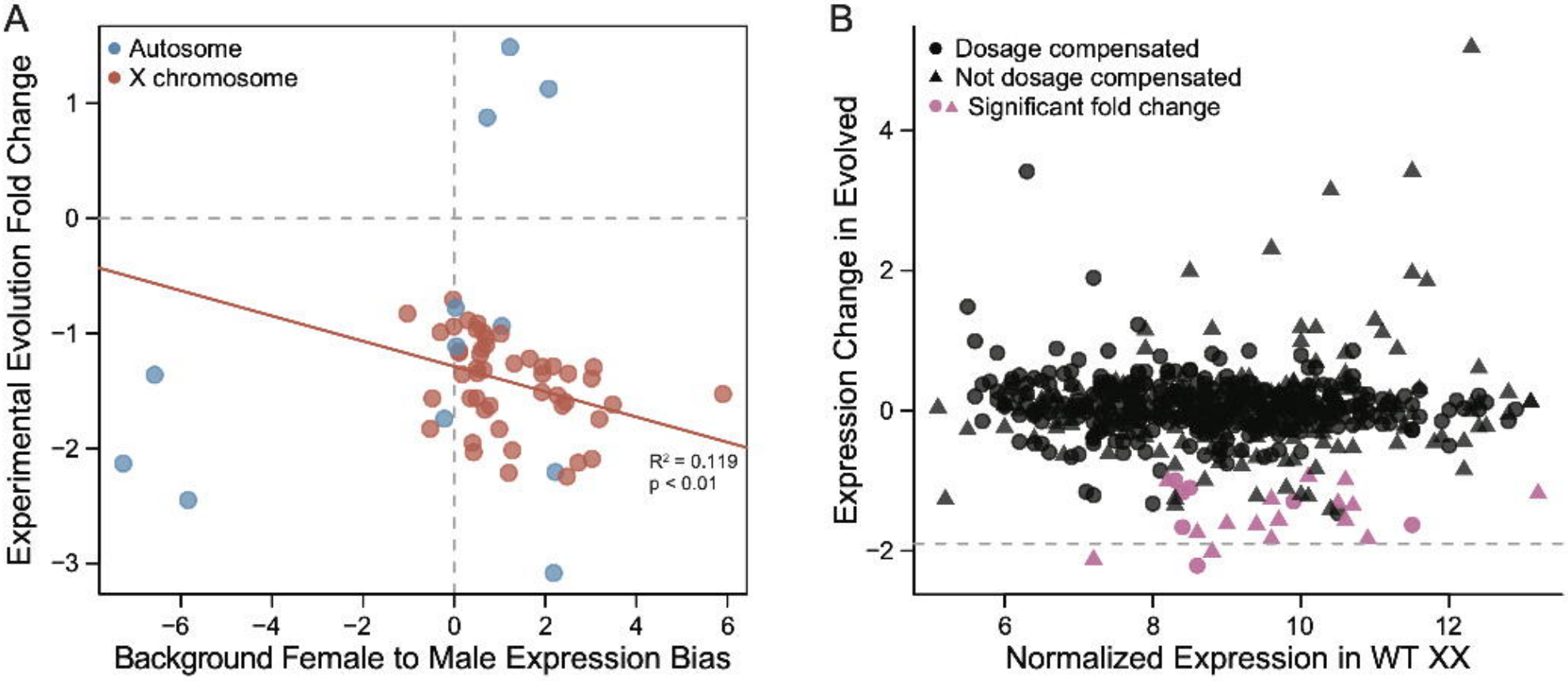
WBGene00022219
lin-15B
lin-15A
WBGene00045418
WBGene00219374


A
Autosome
X chromosome
Experimental Evolution Fold Change
Background Female to Male Expression Bias
R² = 0.119
p < 0.01
B
Dosage compensated
Not dosage compensated
Significant fold change
Expression Change in Evolved
Normalized Expression in WT XX