

1 **Rapid sex-specific adaptation to high temperature in *Drosophila***

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15 **Abstract**

16 The pervasive occurrence of sexual dimorphism demonstrates different adaptive strategies of
17 males and females. While different reproductive strategies of the two sexes are well-
18 characterized, very little is known about differential functional requirements of males and
19 females in their natural habitats. Here, we study the impact environmental change on the
20 selection response in both sexes. Exposing replicated *Drosophila* populations to a novel
21 temperature regime, we demonstrate sex-specific changes in gene expression, metabolic and
22 behavioral phenotypes in less than 100 generations. This indicates not only different functional
23 requirements of both sexes in the new environment but also rapid sex-specific adaptation.
24 Supported by computer simulations we propose that altered sex-biased gene regulation from
25 standing genetic variation, rather than new mutations, is the driver of rapid sex-specific
26 adaptation. Our discovery of environmentally driven divergent functional requirements of
27 males and females has important implications-possibly even for gender aware medical
28 treatments.

29 **Introduction**

30 The ubiquity of sexual dimorphism in dioecious organisms reflects the discordant selection
31 pressure driven by divergent reproductive roles of males and females (Chapman, 2006). For
32 instance, males typically evolve to increase their mating frequency and success of fertilization,
33 while females benefit from better resource allocation to their offspring (Brenzdahl et al., 2018;
34 Civetta and Clark, 2000; Friberg and Arnqvist, 2003). Often, such differential requirements of
35 males and females results in sexual conflict, preventing males and females to reach sex-
36 specific trait optima (Bonduriansky and Chenoweth, 2009; Lande, 1980; Mank, 2017a; Rice,
37 1992). Based on the widespread sexual dimorphism, several models for the evolution of sexual
38 dimorphism from a largely shared genome have been proposed (Barson et al., 2015; Day and
39 Bonduriansky, 2004; Mank, 2017b; Parsch and Ellegren, 2013; Pennell and Morrow, 2013;
40 Rice, 1984; Telonis-Scott et al., 2009). One implicit assumption of these studies is that stable
41 sex-specific fitness landscapes are persisting over long evolutionary time scales. However,
42 ecological changes, such as environmental fluctuations, occur at high rates (Reznick and
43 Ghalambor, 2001). If such environmental factors affect the sex-specific fitness landscapes,
44 sudden ecological changes may impose selection for novel/altered sexual dimorphism in a
45 population (Camus et al., 2019).

46 To date, limited attention has been given to the evolutionary dynamics of sex
47 differences in response to changing environments. The clinal variation of sexual dimorphism
48 for a small number of phenotypes (Blanckenhorn et al., 2006; Chenoweth et al., 2008) and gene
49 expression (Allen et al., 2017; Hutter et al., 2008) in *Drosophila* suggests that sex-specific
50 adaptation in response to environmental heterogeneity is not uncommon. When the
51 requirements of males and females differ in an environment-specific manner, the adaptive
52 response is contingent on the availability of segregating variants with sex-specific or sex-biased
53 effects. Without the corresponding variants, sex-specific adaptation requires new mutations,

54 resulting in slow evolutionary responses. Here, we use experimental evolution for direct
55 experimental evidence that sex-specific adaptation can be triggered by a rapid environmental
56 shift within a few generations.

57

58 **Results and Discussion**

59 **Distinct phenotypic changes of females and males in a novel environment**

60 We explored the phenotypic evolution of males and females by studying gene expression
61 because many of these molecular phenotypes can be scored with a high precision at moderate
62 costs. Furthermore, in contrast to high-level phenotypes, which are typically selected on a
63 priority criteria, the analysis of gene expression is unbiased. We measured gene expression of
64 10 replicate populations which evolved independently for more than 100 generations in a
65 simple and well-controlled high-temperature selection regime (Barghi et al., 2019). In each
66 sex, we screened for genes with parallel changes in expression across the replicated evolved
67 populations compared to their same-sex ancestors. After accounting for allometric changes
68 during evolution (see Methods), we identified 2,366 and 4,151 genes (25% and 44% of all
69 expressed genes, $N=9,457$) showing evolutionary responses in males and females respectively
70 ($FDR < 0.05$; Supplementary File 1 and Fig. 1-Fig. S1). The evolution in gene expression was
71 vastly different between the sexes, resulting in almost uncorrelated gene expression changes
72 (Fig. 1a). Only 760 genes (14%; 469 up-regulated and 291 down-regulated) evolved
73 concordantly in both sexes. 1,295 genes (24%) changed expression specifically in males (657
74 up-regulated and 638 down-regulated) and 3,080 genes (57%) evolved in females only (1,877
75 up-regulated and 1,203 down-regulated). Interestingly, 311 genes (6%) displayed divergent
76 responses to selection in the two sexes (Fig. 1b). The discordant gene expression evolution of
77 males and females indicates different functional requirements in the novel environment.

78 To determine the diverged functional requirements of males and females in the new
79 environment, we tested for enrichment of gene ontology (GO) terms and tissue-specific
80 expression (Fig. 1c and d, Supplementary File 2 and 3). We found a striking pattern of
81 enrichment that suggested sex-specific evolution of fatty acid metabolism in both the GO term
82 and tissue-specific enrichment analyses. Genes highly expressed in fat body tissue were over-
83 represented among the 1,280 genes with upregulation in males, but over-represented among
84 the 1,648 genes with downregulation in females (FET, FDR < 0.01 in both tests, Fig. 1d and
85 Supplementary File 3). GO enrichment analysis of genes with male-specific upregulation
86 further highlighted biological processes like “lipid metabolic process”, “acyl-CoA biosynthetic
87 process”, “fatty acid elongation” and “triglyceride catabolic process” (Supplementary File 2).
88 Similar GO categories were enriched among the 154 antagonistically evolving genes that were
89 upregulated in males but downregulated in females (Supplementary File 2). Interestingly, two
90 apparently counteracting processes, fatty acid synthesis and degradation, were both
91 upregulated in males (Fig. 2a) whereas in females, only genes involved in fatty acid synthesis
92 were significantly downregulated (Fig. 2a). A link between these changes in gene expression
93 and a higher-level phenotype is suggested by the observation that these laboratory populations
94 experienced a significant decrease of fat content only in females but not in males (Barghi et
95 al., 2019) (Fig. 2b).

96 In addition, sex-specific responses to selection in gene expression were also related to
97 neuronal signaling. The evolution of dopamine signaling during temperature adaptation has
98 previously been reported in male flies of the same population (Jakšić et al., 2019). The 1,086
99 genes that evolved decreased expression in males were enriched in brain and ganglion tissues
100 (FET , FDR < 0.001 in both tests; Fig. 1d and Supplementary File 3) whereas there was no
101 enrichment in these tissues for females. Likewise, gene expression of dopaminergic processes
102 (e.g.: *Ddc*, *DAT* and *Dop1R2*) evolved downregulation in males but did not evolve in females

103 (Fig. 2c). In contrast, only females evolved increased expression of genes involved in
104 octopamine biosynthesis and signaling (e.g.: *Tdc1*, *Tdc2* and *Octα2R*) (Fig. 2c).

105 The sex-specific modulation of transcriptional activity in different neuronal circuits
106 may trigger changes in sex-specific fitness-related behaviors such as male courtship and female
107 oviposition. In support of this hypothesis, the GO terms “copulation” and “male courtship
108 behavior” were enriched among the 154 antagonistic genes up-regulated in males, as was
109 “oviposition” among the 1,877 genes with female-specific up-regulation (Supplementary File
110 2). The increased fecundity of evolved females (Barghi et al., 2019) fits the expectations for
111 increased octopamine synthesis (Cole et al., 2005; Monastirioti, 2003). Female fecundity is,
112 however, a complex trait which may be affected by many factors other than increased
113 octopamine level. We tested therefore another octopamine-related phenotype that was not
114 selected in the experiment, ovarian dormancy in response to cold temperatures (Andreatta et
115 al., 2018). Confirming the increased octopamine level in the evolved females, dormancy
116 incidence was lower at two different dormancy-inducing temperatures (10 and 12°C) (Fig. 2d
117 and Fig. 2-Fig. S1). Further, we also observed changes in male-specific behavior after 100
118 generations of adaptation; evolved males spent more time chasing females and made more
119 copulation attempts than ancestral ones (Fig. 2e and Fig. 2-Fig. S2).

120 The sexually discordant evolution of several phenotypes, including gene expression,
121 metabolism and behavior, provides evidence that sex-specific adaptive processes occurred in
122 experimental populations exposed to a novel temperature regime. This raises the important
123 question of how potentially conflicting selection pressures on the shared genome have been
124 decoupled during 100 generations of evolution.

125

126 **Rapid sex-specific adaptation can be driven by altered sex-biased gene regulation**

127 Sexually dimorphic gene expression is abundant in *Drosophila* (Parsch and Ellegren, 2013)
128 and 95% of the genes in the ancestral population of this study are also sex-biased
129 (Supplementary File 1). This implies the decoupling of selection on the gene expression in
130 males and females (Mank, 2017a) as well as the presence of a sex-biased regulatory
131 architecture of the transcriptome (Mank, 2017b; Parsch and Ellegren, 2013; Pennell and
132 Morrow, 2013) in the ancestral population. Transcription factors (TF) with sex-bias in
133 expression or splicing are the key factor underlying this sex-biased regulatory architecture
134 (Mank, 2017a). It has been hypothesized that relatively fast sex-specific responses to
135 discordant selection may be driven by fixation of novel mutations resulting in sex-biased gene
136 expression (Stewart et al., 2010; Van Doorn, 2009). However, we observe sex-specific
137 expression changes across replicates after only 100 generations, in which case de novo
138 mutations in individual replicates are unlikely to be the driver (Burke et al., 2010). Rather,
139 selection on standing genetic variation in existing sex-specific genetic architecture seems the
140 most likely mechanism allowing replicated populations to approach different functional
141 requirements of the two sexes in the new environment over such a short timescale (Fig. 3).

142 Candidate TFs supporting this hypothesis would regulate both genes with sex-biased
143 expression (criterion 1) and genes with a significant evolution of sex-bias in expression
144 (criterion 2). Furthermore, the sex-bias of these TFs must have evolved in a direction
145 compatible with the changes of their target genes (criterion 3). Of 656 annotated TFs expressed
146 in our populations, 300 TFs evolved a change in sex-biased expression (i.e. either evolve a new
147 sex bias or the ancestral sex bias changes); 210 and 80 evolved either in females or males,
148 respectively, and 10 changed in opposite direction in the two sexes (Supplementary File 4).
149 Based on cis-regulatory element enrichment, we identified 69 TFs which regulate genes with
150 sex-biased expression and a total of 198 TFs that target genes with sex-bias evolving in
151 opposite direction (Supplementary File 5). In the end, 19 TFs satisfied all our three criteria for

152 the most likely candidates targeted by the discordant selection in the two sexes (Supplementary
153 File 6). Despite genomic time series data being available for these populations (Barghi et al.,
154 2019), extensive linkage structure in the populations preclude an unambiguous identification
155 of selected TF alleles. Future functional studies will show which of these candidate TFs are
156 accomplishing the decoupling of male and female requirements and which molecular processes
157 contribute to adaptation of the two sexes in a novel temperature regime. Nevertheless, we
158 caution that the evolution of gene expression is most likely polygenic, with several-or even
159 many loci contributing to the evolution of sex-bias. In this case, both genomic responses and
160 functional tests may be complicated due to the expected small effects of individual loci.

161 Using computer simulations, we further corroborated the hypothesis that sex-specific
162 adaptation can be achieved rapidly in the presence of segregating regulatory variants which
163 alter the sex-bias of a trait. Based on the haplotype information of the founder lines initiating
164 the experiment (Barghi et al., 2019), we simulated traits (expression value) each controlled by
165 50 additive loci (TFs) of which 0, 1, 2, 5, 10 or 20 are sex-specific (effect size = 0 in one
166 sex)/sex-biased (2-fold difference in effect size). The simulated populations were exposed to a
167 selection regime where males and females of the same population have different fitness optima
168 for the focal trait and we monitored the phenotypic change in each sex during 100 generations.
169 100 simulations were performed under each scenario. Without sex bias in the effect size ($r_{mf} =$
170 1), neither males nor females could respond to the discordant selection (Fig. 4). With 40% of
171 the loci contributing to the trait being sex-specific ($r_{mf} = 0.49 \pm 0.2$) or sex-biased ($r_{mf} =$
172 0.87 ± 0.05), both males and females can evolve toward the opposing optima (Fig. 4 and Fig. 4-
173 Fig. S1). Nevertheless, sex-specific or sex-biased expression is not required for many
174 contributing loci. Already two sex-specific ($r_{mf} = 0.94 \pm 0.08$) loci significantly decouple the
175 response of the two sexes (Fig. 4b) under opposing selection pressures.

176

177 **Maintenance of genetic variation with sex-biased effects**

178 As discussed above, the rapid sex-specific responses, which are highly parallel across
179 replicates, in combination with the gain and loss of sexual dimorphism (Fig. 1-Fig. S2)
180 highlight the importance of standing genetic variation in sex-biased regulatory architecture.
181 This raises the interesting question of how genetic variation with sex-biased effects is
182 maintained. Assuming a simple genetic basis and a stable fitness landscape with pronounced
183 differences between the two sexes, alleles with dimorphic expression are expected to become
184 fixed. We propose two, not mutually exclusive hypotheses to explain the discrepancy to our
185 observation. First, the fitness landscape of some sex-specific phenotypes could vary in response
186 to environmental fluctuation. In this case, alleles controlling the sex difference of a trait could
187 be segregating and maintained in a population. As natural *Drosophila* populations regularly
188 encounter seasonal temperature fluctuations, candidate alleles regulating sex-specific
189 temperature adaptation can be maintained at sufficiently high frequencies to facilitate rapid
190 responses. The impact of seasonal variation on oscillating allele frequency changes has been
191 recently described experimentally and theoretically (Bergland et al., 2014; Wittmann et al.,
192 2017). The second hypothesis assumes a polygenic basis. We note that unambiguous sex-
193 limited modifiers (e.g.: male and female isoforms of *doublesex* (Kopp et al., 2000)) do not
194 preclude polygenic adaptation - these sex-limited modifiers may regulate many down-stream
195 regulators that respond to the environmental change. Thus, already minor frequency shifts of
196 these down-stream regulators could mediate the observed evolution of sex-specific gene
197 expression changes. Importantly, under polygenic adaptation segregating variation is
198 maintained for rather long time-scales (Barton and Keightley, 2002; Gillespie, 1984; Gillespie
199 and Turelli, 1989). Indirect support for this hypothesis comes from the observation that no
200 significant SNPs explaining the sex difference for multiple human traits can be identified
201 (Randall et al., 2013). Under this scenario, rapid evolution of the sex difference may be

202 achieved by the heterogeneous genotypic changes across replicated populations (Barghi et al.,
203 2019).

204

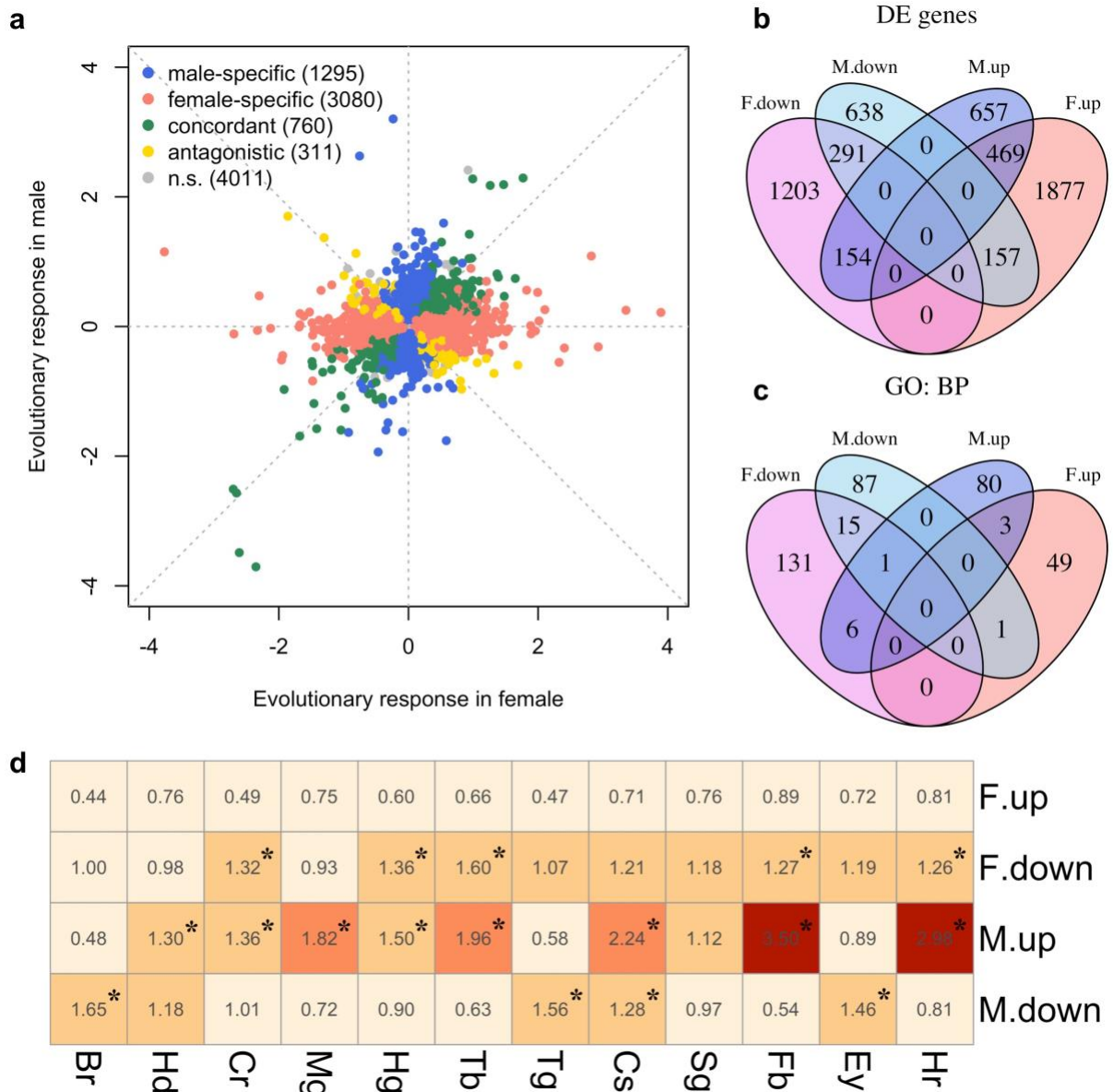
205 **Conclusion and outlooks**

206 This study demonstrates the power of experimental evolution to study sex-specific adaptation
207 after an environmental shift. A substantial fraction of the transcriptome and related high-level
208 phenotypes rapidly developed discordant changes in the two sexes upon exposure to a new
209 environment. We propose that variation segregating in the ancestral population has facilitated
210 the evolution of sex-biased gene expression, which in turn provides the basis for the sex-
211 specific adaptation evidenced by the broad range of phenotypes evolving in different directions
212 in males and females.

213 While we provided robust evidence for sex-specific adaptation, it is important to keep
214 in mind that the identification of the selected traits in both sexes is an extremely challenging
215 task. While 60% of genes changed expression in a sex-specific manner, it is unlikely that each
216 of them is independently selected. We can anticipate many ways how the sex specific
217 phenotypic changes have been achieved, ranging from allometric changes during adaptation to
218 selection acting on cis-regulatory variation of highly pleiotropic transcription factors. Further
219 characterization of the adaptive changes needs to distinguish between two goals. One goal,
220 which is pursued in many studies, is the identification of the gene(s) that experienced a
221 frequency change of a favored variant as contribution to the adaptive phenotype. The other
222 goal, is the identification of the selected phenotype. Given the pleiotropic effects of many
223 genes and the polygenicity of most adaptive phenotypes (Barghi et al., 2019; Pritchard et al.,
224 2010), it is apparent that the characterization of individual selected alleles has clear limitations
225 in reaching the second goal. In fact, the justification of studies aiming to characterize adaptive

226 allelic variants has been challenged (Rockman, 2012). More rewarding would be the
227 characterization of the adaptive trait, which is selected in a sex-specific manner. Our
228 enrichment analysis and characterization of high-level phenotypes aimed towards this
229 direction, but we cannot distinguish between correlated phenotypic changes and the actual
230 selected phenotypes.

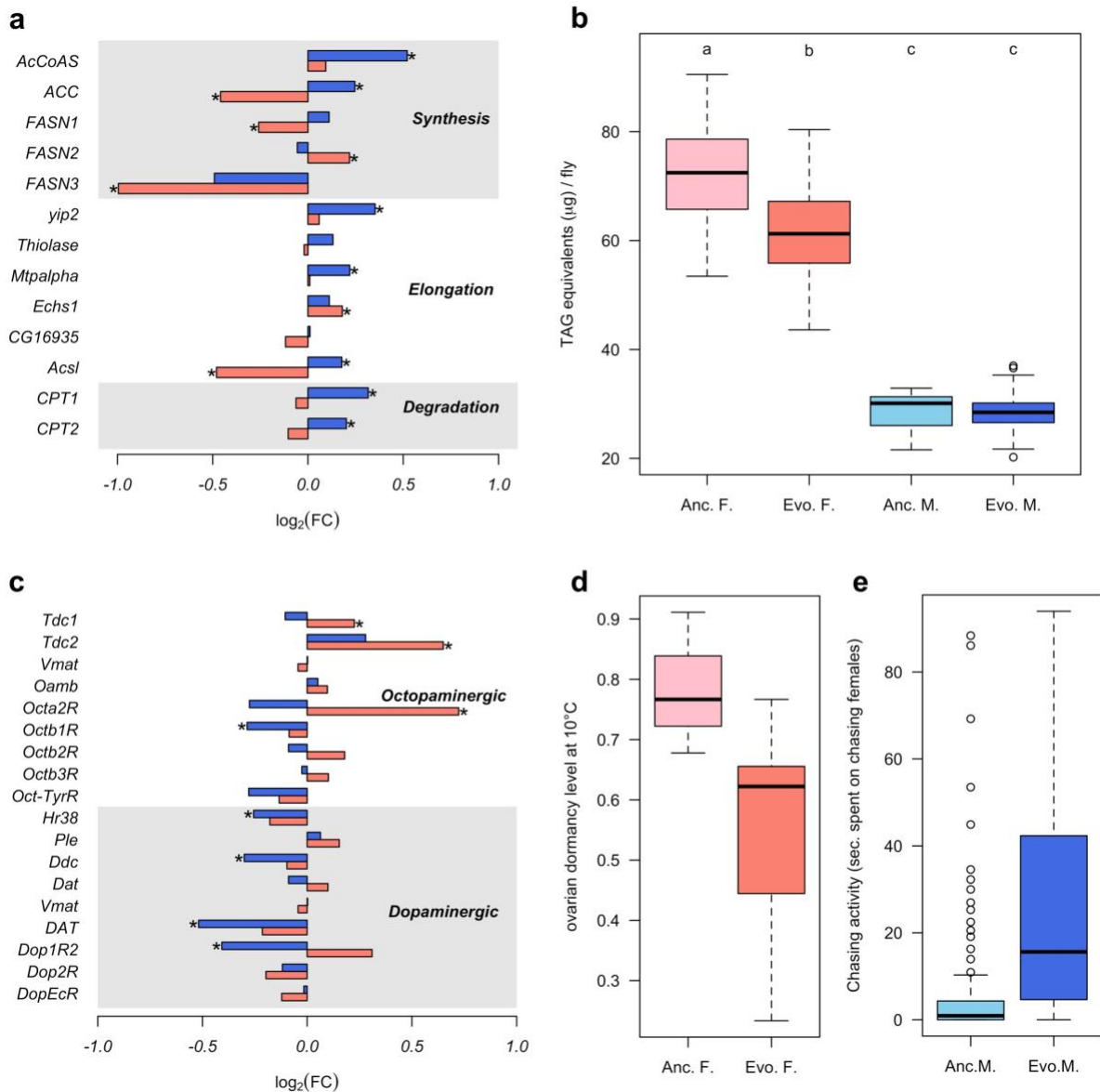
231 While most of this report focused on the rapid evolution of sex-specific adaptation, the
232 driving forces behind this have not been discussed to the same extent, largely because they will
233 require further functional characterization. Nevertheless, in line with sex-dependent dietary
234 effects on fitness (Camus et al., 2019), the fact that males and females have vastly different
235 functional requirements after being exposed to a novel environment has far reaching
236 consequences-well beyond *Drosophila*. We anticipate that our results will have profound
237 influence on biomedical research and medical treatments which need to account for the
238 overwhelming differences of the two sexes in particular with respect to new environmental
239 stressors, reaching from diet to climatic conditions.



240

241 **Fig. 1. Sex-specific gene expression evolution adapting to a high temperature.** **a.** Evolution
 242 of gene expression in females (x axis) and males (y axis). The evolutionary changes of all
 243 expressed genes are shown on log₂ scale. Genes showing different patterns of evolution are
 244 highlighted in different colors. **b.** The majority of the genes with significant expression changes
 245 is sex-specific. Venn diagram showing the number of genes with significantly different gene
 246 expression patterns (DE: Differential Expression; M.up/F.up: males/females evolved higher
 247 gene expression, M.down/F.down: males evolved lower gene expression). **c.** Genes with
 248 evolved expression changes in males and females are involved in nearly mutually exclusive

249 sets of biological processes. Venn diagram of sets of GO (biological processes) terms enriched
250 by the genes changing their expression for each direction in each sex (i.e. four sets of candidate
251 genes: up/down-regulation in males/females). For instance, there are only 3 biological
252 processes repeatedly found among the 90 and 53 processes involving up-regulated genes in
253 males and females respectively. **d.** The tissue enrichment of genes significantly evolving for
254 either direction in males and females (Br-brain, Hd-head, Cr-crop, Mg-midgut, Hg-hindgut,
255 Tb-malpighian tubule, Tg-thoracoabdominal ganglion, Cs-carcass, Sg-salivary gland, Fb-fat
256 body, Ey-eye and Hr-heart). Each cell represents the result of a Fisher's exact test. The colors
257 and numbers denote the magnitude of odds ratio and statistical significance ($FDR < 0.05$) is
258 indicated with *. Consistent with GO enrichment results, gene expression evolution in males
259 and females may occur in different tissues.



260

261 **Fig. 2. Sex-specific phenotypic evolution. a. and c.** Genes involved in fatty acid metabolism

262 and monoaminergic neural signaling evolve in response to high temperature. The evolutionary

263 changes in males (blue bar) and females (red bar) are shown on \log_2 scale. Statistical

264 significance ($\text{FDR} < 0.05$) is indicated with *. For both set of genes, the evolution is largely

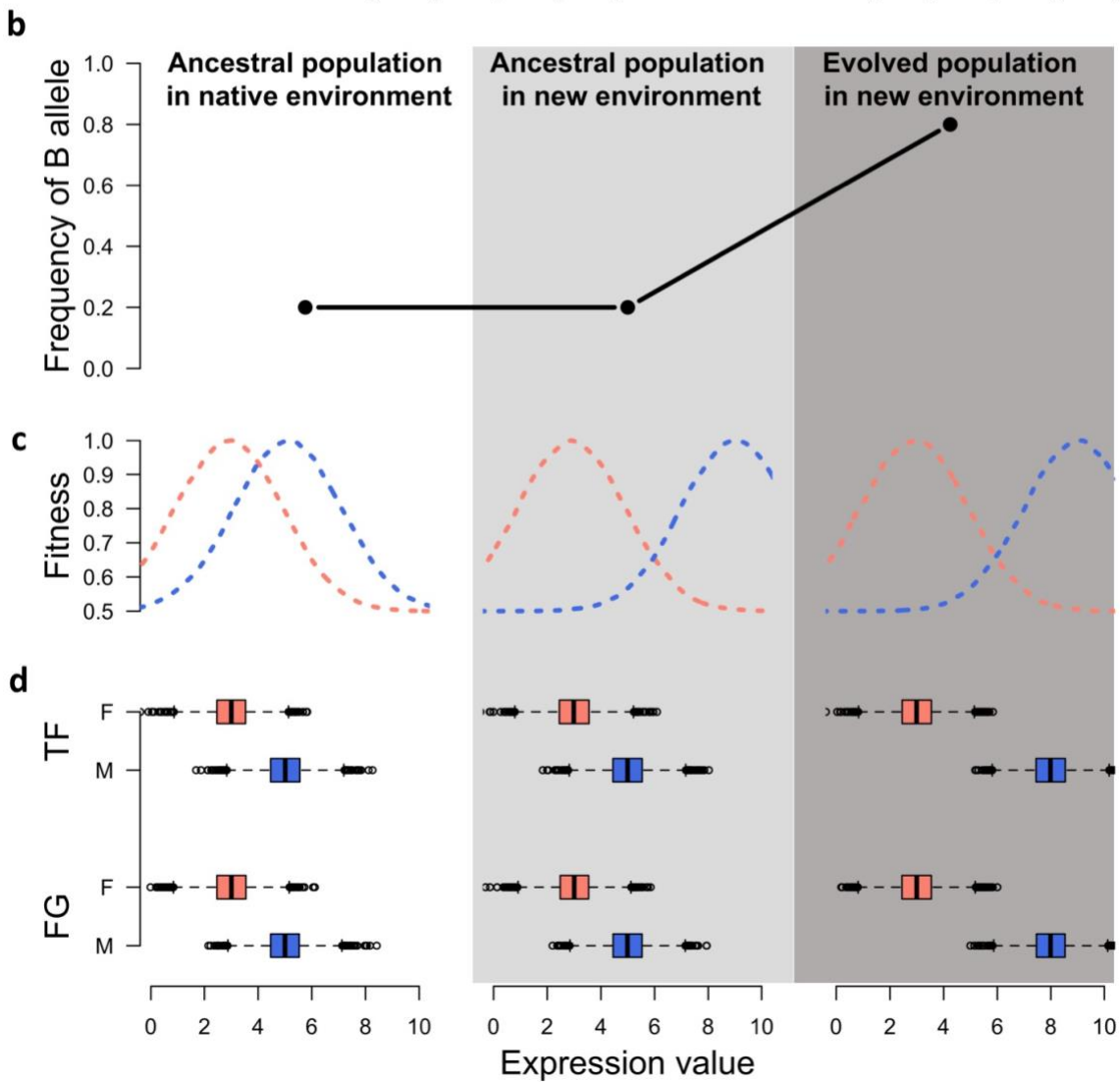
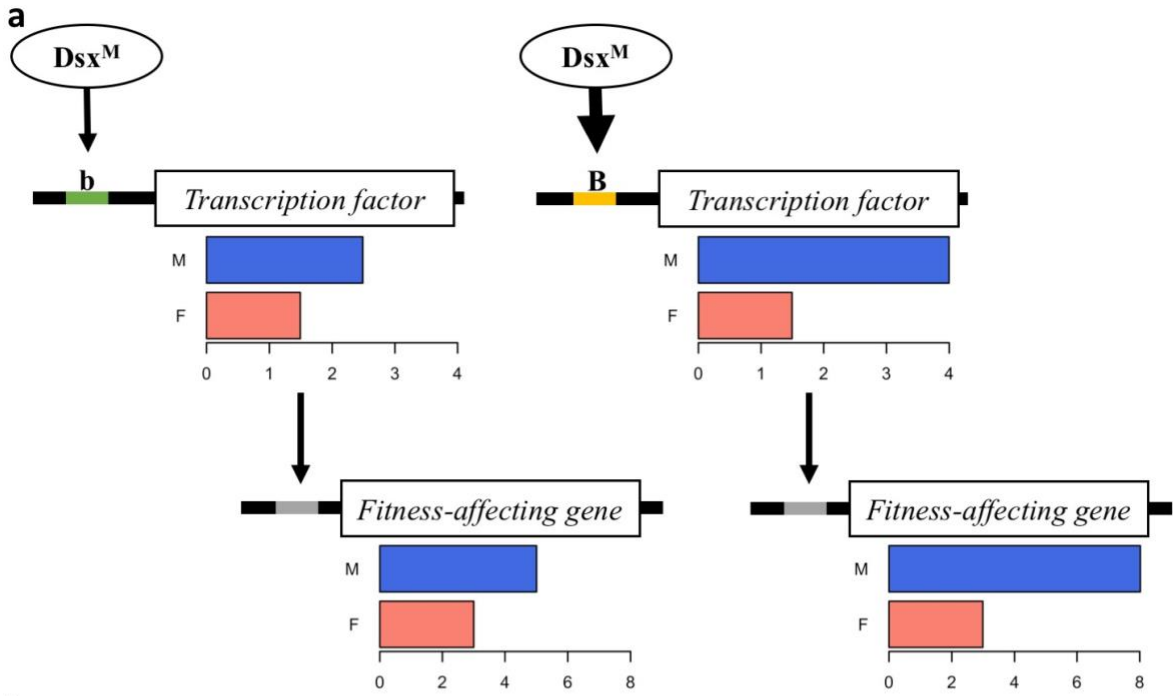
265 sex-specific or even sexually discordant. **b.** Level of triglycerides, the main constituent of body

266 fat (data from (Barghi et al., 2019)). Evolved females have significantly lower fat content than

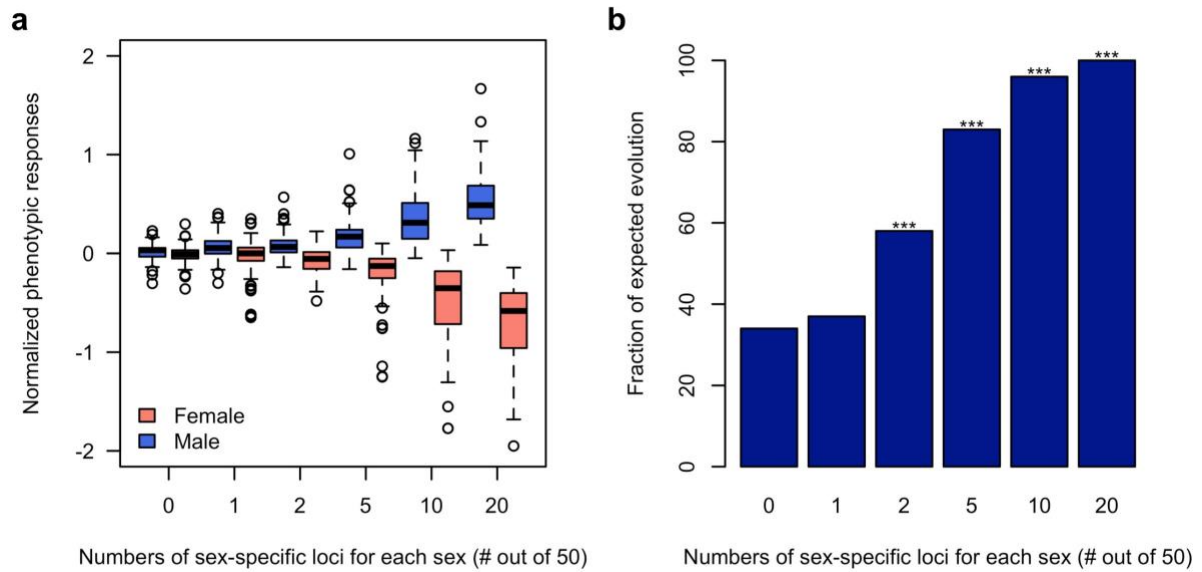
267 the ancestral ones. No significant difference is found in males. Two-way ANOVA and

268 Tuckey's HSD test. **d.** Ovarian dormancy incidence at 10°C in ancestral and evolved females.

269 Evolved females have a lower dormancy incidence than ancestral ones (Wilcoxon's test, $W =$
270 1.5, $p = 0.028$). **e.** Time males chasing females. Evolved males spent significantly more time
271 chasing females (Wilcoxon's test, $W = 1323.5$, $p < 0.001$).



273 **Fig. 3. A simple model for rapid evolution of sex-specific adaptation.** Regulatory variation
274 segregating at a transcription factor is selected for a more pronounced difference in gene
275 expression between sexes. This also causes more pronounced expression differences in a
276 downstream gene satisfying the altered requirements of the two sexes in the new environment.
277 **a.** Regulatory cascade of a transcription factor (TF) controlled by sex-specific isoforms of Dsx.
278 Two alleles with different binding affinity ($B > b$) with DsxM but not with DsxF are regulating
279 downstream genes affecting fitness (FG). **b.** Frequency of the allele increasing sex-bias (B
280 allele) at three different stages: in the native (natural) environment, in the new hot environment
281 at the start of the experiment, in the new hot environment at the end of the experiment. **c.**
282 Fitness landscape at the three different stages. **d.** Expression of TF and FG in males and females
283 at the different stages. After 100 generations, the frequency increase of the allele increasing
284 sex-biased expression of the TF results in a resolved intra-locus conflict.



285

286 **Fig. 4. Rapid decoupling of the phenotypic response to sexually discordant trait optima**

287 **by a few sex-specific loci. a.** The phenotypic response of a trait controlled by 50 loci after 100

288 generations of sexually discordant selection. Different numbers of sex-specific loci in each

289 sex are shown. For each scenario, 100 independent computer simulations were performed. The

290 normalized phenotypic change is calculated as the ratio between phenotypic change and

291 phenotypic variance of the ancestral population. **b.** Fraction of simulations for which the focal

292 trait increases in males but decreases in females. The statistical significance denoted by an

293 asterisk is based on one-sample proportion test comparing to the control simulation without

294 any sex-specific locus. Bonferroni's correction is applied. Already two sex-specific loci in each

295 sex significantly decouples the phenotypic responses to the discordant selection. With

296 increasing numbers of sex-specific loci, the difference between the sex-specific phenotypic

297 responses becomes more pronounced.

298 **Methods**

299 Experimental evolution

300 The set-up of the experimental evolution populations is described in (Barghi et al., 2019). In
301 brief, 10 replicated outbred populations were constituted from 202 isofemale lines derived from
302 a natural *Drosophila simulans* population collected in Tallahassee, Florida, USA in 2010.
303 Replicated populations have been independently adapting to a laboratory environment at
304 18/28°C with 12h dark/12h light photoperiod for more than 160 generations with a census
305 population size of 1000-1250 adults per population per generation.

306

307 RNA-Seq common garden experiment

308 The collection of samples for RNA-Seq and all other phenotypic assays, was preceded by two
309 generations of common garden rearing. Two different RNA-Seq data sets were generated for
310 this study: The first one, in which highly replicated whole body samples were collected,
311 represents the main dataset that we used to contrast gene expression levels of females and males
312 from ancestral and hot evolved populations. The second one with gonads and carcass being
313 analyzed separately was generated to correct for allometric differences between evolved and
314 ancestral populations.

315 The first data set comes from a common garden experiment (CGE) performed after 103
316 generations of evolution in the hot environment and this CGE has been described in (Barghi et
317 al., 2019; Hsu et al., 2019; Jakšić et al., 2019). We reconstituted five replicates of the ancestral
318 population from 184 founder isofemale lines by generating five pools with five mated females
319 from each isofemale line. No significant allele frequency differences are expected between the
320 reconstituted ancestral populations and the original ancestral populations initiating the
321 experiment (Nouhaud et al., 2016). Because we evaluated phenotypes on the population level,
322 even deleterious mutations will have a very limited impact, because they occur only in a single

323 isofemale line, which represents a very small fraction of the total population. For each of the
324 10 hot evolved replicates, we generated three sub-replicates by multiple egg lays. The five
325 ancestral replicates and all hot evolved sub-replicates were reared in common garden for two
326 generations with controlled low egg density (400 eggs/bottle) in the same temperature regime
327 as during the evolution experiment. After two generations under CGE conditions, flies were
328 collected from each replicate/sub-replicate a few hours after eclosion and maintained on fresh
329 food under the 18/28°C temperature regime to allow for mating. On the third day after eclosion,
330 sexes were separated under CO₂ anesthesia and allowed to recover for two days. At the age of
331 five days, 50 flies of each sex were snap frozen in liquid nitrogen at 2pm and stored at -80°C
332 until RNA extraction. We sequenced the transcriptomes of 50 females and males from each of
333 the five ancestral replicates and from each of the 10 hot evolved replicates with three sub-
334 replicates each for males and two sub-replicates for females. The third sub-replicate of the hot
335 evolved female samples was frozen at a different age which prevented the joint analysis in the
336 context of this study (Hsu et al., 2019).

337 The second RNA-Seq data set was generated at generation 140 of the hot evolving
338 populations to correct for potential differences in the relative size of gonadal and carcass tissue
339 between ancestral and evolved populations. CGE set-up and maintenance were repeated as
340 described above, without sub-replication of the hot evolved replicates: 50 whole body samples
341 for females and males were collected from five reconstituted ancestral and all 10 hot evolved
342 replicates and snap-frozen at the age of five days at 2pm. Gonadal and carcass tissue was
343 sampled from six reconstituted ancestral and six randomly chosen hot evolved replicates
344 (replicates no. 1, 4, 5, 6, 8, 9). For each replicate, 50 female and 50 male flies were dissected
345 in PBS at the age of 5 days and dissected gonadal tissues and remaining carcasses were
346 immediately preserved in Qiazol and stored at -80°C.

347

348 RNA extraction and library preparation

349 Total RNA was extracted using the same procedure for all samples: homogenized in Qiazol
350 with a pestle. Total RNA was extracted from the homogenate using the Qiagen RNeasy
351 Universal Plus Mini kit (Qiagen, Hilden, Germany) with DNase treatment to remove traces of
352 genomic DNA. Libraries were prepared on the Neoprep Library Prep System (Illumina, San
353 Diego, USA) starting from 100ng total RNA and following the manufacturer's recommended
354 protocol for the TruSeq stranded mRNA Library Prep Kit for Neoprep. Neoprep runs were
355 performed using software version 1.1.0.8 and protocol version 1.1.7.6 with default settings for
356 15 PCR cycles and an insert size of 200bp. Libraries were arranged in randomized order on
357 library cards. To avoid batch effects, we used library cards with the same lot number for all
358 samples for which direct comparisons of expression levels were planned (lot no. 20123465:
359 CGE at generation 103, males, whole body, all ancestral and hot evolved samples; lot no.
360 20173962: CGE at generation 103, females, whole body, all ancestral and hot evolved samples;
361 lot no. 20182049: CGE at generation 140, females and males, whole body and gonadal tissue).
362 50bp single-end reads were sequenced on an Illumina HiSeq 2500.

363

364 RNA-Seq data processing

365 All sequencing reads were trimmed with ReadTools (Version: 1.5.2) (Gómez-Sánchez and
366 Schlötterer, 2018) based on a quality score of 20, and mapped with GSNAP (Version: 2018-
367 03-25; Parameters: -k 15 -N 1 -m 0.08) (Wu and Nacu, 2010) to *Drosophila simulans* reference
368 genome (Palmieri et al., 2015) (Supplementary File 7). Exon-aligned reads were counted with
369 Rsubread (Version: 1.30.9) (Liao et al., 2013) based on the annotation (Palmieri et al., 2015)
370 and the expression level of each gene was quantified after normalizing the exon-aligned read
371 counts by TMM method implemented in edgeR (Version: 3.22.5) (Robinson et al., 2010). Only

372 genes with more than 0.1 count per million base pairs in each sample of the main dataset (1st
373 CGE) were retained for the analysis to avoid biased analyses.

374

375 Estimation and correction of the allometric difference

376 Using an independent CGE that consisted of dissected samples (2nd CGE, correcting dataset),
377 we corrected for potential differences in the relative size of gonadal and remaining carcass
378 tissues in ancestral and hot evolved populations for each gene.

379 For each gene, we formulated its average expression across whole-body samples
380 ($\overline{y_{wb,i}}$) with the average expression across gonad samples ($\overline{y_{g,i}}$) and carcass samples ($\overline{y_{c,i}}$) as:
381 $\overline{y_{wb,i}} = \alpha_i \overline{y_{g,i}} + (1 - \alpha_i) \overline{y_{c,i}}$, where α is the coefficient measuring the relative portion of
382 gonadal expression of a gene in whole body expression, ranging from 0 to 1 (Method-Fig. S1).
383 If a gene is expressed at similar level in both gonadal and somatic tissues, it would not be
384 affected by differences in tissue scaling. We excluded these genes in the comparison of tissue-
385 scaling and applied no correction for them in the subsequent analysis. Leave-one-out cross
386 validation was performed to evaluate the accuracy and robustness of the method. The
387 estimation of the scaling coefficients for each gene was robust (Supplementary File 8). In
388 addition, the prediction was nearly perfect (Supplementary File 9).

389 Comparing the distribution of gene-wise estimates of scaling coefficients, we found
390 significant difference between ancestral and evolved populations for both sexes (Kolmogorov-
391 Smirnov test $D = 0.18$ and 0.12 for females and males, respectively; $p < 0.001$ in both tests;
392 Method-Fig. S2). This suggested that the gonad-carcass size ratio may have significantly
393 changed during the adaptation to the new environment. A proper correction is necessary for
394 unbiased inference. Hence, we normalized the tissue-scales of each ancestral sample to the
395 scale of evolved samples. We reconstructed pseudo whole-body samples using the expression

396 data of dissected samples of the ancestral populations and scaling coefficients estimated from
397 the evolved samples as: $y_{Wb,i}^{pseudo} = \hat{\alpha}_i^{evo} y_{g,i} + (1 - \hat{\alpha}_i^{evo}) y_{c,i}$.

398 Finally, the ratio of expression levels between the reconstructed pseudo whole-body
399 samples and the original ones ($\frac{y_{Wb,i}^{pseudo}}{y_{Wb,i}}$) for each gene were calculated as the correcting factors
400 ($\hat{\gamma}_i$). Gene-wise correction was applied to ancestral whole-body samples from the 1st CGE by
401 multiplying the expression value of each gene to corresponding $\hat{\gamma}_i$. The corrected samples were
402 used in all subsequent analyses.

403

404 Differential expression (DE) analysis

405 After correction, we modeled the effects of sex and evolution on gene expression variation as:
406 $Y = group + \varepsilon$, where Y is the normalized expression values; $group$ indicates the
407 combination of evolution and sex difference with four levels (ancestral females, ancestral
408 males, evolved females and evolved males) and ε is the random error. Likelihood ratio tests
409 implemented in edgeR were used to perform differential expression analysis on three contrasts:
410 (1) *female evolution*: evolved females vs. ancestral females, (2) *male evolution*: evolved males
411 vs. ancestral males and (3) *sex-bias*: females vs. males. Benjamini and Hochberg's FDR
412 correction (Benjamini and Hochberg, 1995) was applied with the significance threshold of
413 $FDR < 0.05$. Genes showing distinct evolutionary patterns were classified based on criteria in
414 Supplementary File 2.

415

416 Enrichment analysis

417 Gene ontology (GO) enrichment was performed using the default "weight01" algorithm
418 implemented in topGO (version 2.32.0) (Alexa et al., 2006). Genes highly expressed in each
419 tissue were identified based on the FlyAtlas expression dataset (Chintapalli et al., 2007)

420 (required > 2 fold higher expression in a certain tissue than whole-body, Supplementary File
421 3). Fisher's exact test was applied for the enrichment of tissue expression. Except for the GO
422 enrichment analysis of which the method already accounts for multiple testing (Alexa and
423 Rahnenführer, 2018), Benjamini and Hochberg's FDR correction (Benjamini and Hochberg,
424 1995) was applied to account for multiple testing.

425

426 Cis-regulatory element enrichment analysis

427 Enrichment of cis-regulatory elements (CREs) 5kb upstream and intronic sequences of the
428 genes of interest (Supplementary File 5) was identified with RcisTarget (version 1.0.2) (Aibar
429 et al., 2017). We searched for enriched motifs using the latest motif ranking file of *Drosophila*
430 species ("*dm6-5kb-upstream-full-tx-11species.mc8nr.feather*", accessed on 2019-04-08) with
431 parameters, nesThreshold = 3 and aucMaxRank = 1%. Transcription factors (TFs) annotated
432 to bind on the enriched CREs were considered as candidate master TFs regulating the genes of
433 interest.

434 We performed cis-regulatory element enrichment analysis on female-biased, male-biased,
435 female-specifically up-regulated, down-regulated, male-specifically up-regulated, down-
436 regulated, and two sets of antagonistically evolving genes separately (Supplementary File 5).

437

438 Male reproductive activity assays

439 We measured the reproductive activity of five reconstituted ancestral populations and five
440 randomly selected hot evolved replicates at generation 140. After two generations reared in a
441 common garden condition (18/28°C cycling), 10 five-day-old mated males and females from
442 each population were placed together in an agar-based arena (4% agar, 4% sugar) and filmed
443 for 15 minutes at 20 FPS (frame-per-second) at 28°C using the FlyCapture2 system (PointGrey,
444 Version 2.13.3.31). In total, 10 video each for reconstituted ancestral and evolved populations

445 were filmed. The movement and behavior of each fly was tracked using flytracker (Version
446 1.0.5) (Eyjolfsson et al., 2014). Videos that failed the tracking process were not used for
447 subsequent analysis. Janelia Automatic Animal Behavior Annotator (JAABA, Version
448 0.6.0_2014a) was used to annotate and recognize the chasing and attempted copulation
449 behavior (Kabra et al., 2012). We imported the output files of JAABA into R for data
450 processing and statistical analysis. The time a male fly spent on chasing and copulation attempt
451 females was quantified. Wilcoxon's rank sum test was applied to test the difference in
452 reproductive activity of male flies in evolved and ancestral populations.

453

454 Female reproductive dormancy assays

455 We screened three replicates of the reconstituted ancestral and 10 replicated evolved
456 populations for dormancy incidence at generation 167. Ancestral and evolved populations were
457 kept at the same temperature regime for four generations before freshly eclosed female flies
458 were collected within two hours post-eclosion and kept under dormancy-inducing conditions
459 (10°C and 12°C, LD 10:14) for three weeks before dissection. 90 flies from each population
460 and temperature regime were dissected and their oogenesis progression was examined. Each
461 fly was classified as dormant or non-dormant (Lirakis et al., 2018). Wilcoxon's rank sum test
462 was applied to test the difference in dormancy level of female flies in evolved and ancestral
463 populations.

464

465 Simulation

466 We performed forward simulations using *qff* function implemented in MimicrEE2 (v208)
467 (Vlachos and Kofler, 2018). Starting with 189 founder haplotypes (Barghi et al., 2019), in each
468 sex, we simulated a trait controlled by a varied number of loci (0, 1, 2, 5, 10, 20) conferring
469 sex-specific or sex-biased effects while the total number of contributing loci in each sex was

470 constantly 50. For each trait, we assumed an additive model ($a \sim \Gamma(0.5, 2.5)$) and relatively high
471 heritability ($h_2 = 0.8$). A sex-specific locus confers additive effect on a trait in one sex but no
472 effect in the other sex while a sex-biased locus is assumed to contribute to the trait in both
473 sexes but there is a 2-fold difference in its additive effect between the two sexes. Sexually
474 discordant selection, where the trait optimum is shifted three units (i.e. on average, 1.9
475 phenotypic standard deviations) to the left and to the right for males and females respectively,
476 was imposed to the simulated traits for 100 generations assuming balanced sex-ratio. In total,
477 we performed 100 independent simulations for each of the six scenarios in this study. Then,
478 we measured the normalized phenotypic responses to the selection as $\frac{\Delta \bar{p}_{100-0}}{\sigma_0^2}$, where $\Delta \bar{p}_{100-0}$
479 is the mean phenotypic difference between F100 and F0 of the populations and σ_0^2 is the
480 phenotypic variance when the experiment starts. We calculated the fractions of simulations in
481 which expected phenotypic responses in the two sexes (increase in males but decrease in
482 females) were observed. One-sample proportion test was performed to test for significant
483 difference between each scenario to the control group. Bonferroni's correction was applied to
484 account for multiple testing.

485

486 Data availability

487 Sequence reads from this study will be available from the European Sequence Read Archive
488 (<http://www.ebi.ac.uk/ena/>) under the study accession number PRJEB35504 and PRJEB35506.

489 Original data and scripts for the analysis could be found as supplementary files or on the github
490 repository of this study (https://github.com/ShengKaiHsu/Dsim_sex-specific_adaptation).

491

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498

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500

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678

679

680 **Titles and legends for figure supplements**

681 **Fig. 1-Fig. S1. Parallel responses of adaptive genes across replicates.**

682 Normalized expression of significant genes identified in males (A) and females (B). The heat
683 color indicates the expression of each gene in each sample. Comparing ancestral samples (B01-
684 B05) to each of the evolved samples (H01-H10), 91% and 87% of the candidate genes in males
685 and females change their expression to the same direction in all replicates, respectively.

686

687 **Fig. 1-Fig. S2. Evolution of sexual dimorphism.**

688 During the adaptation to the hot laboratory environment, 673 ancestrally unbiased genes
689 evolved to exhibit significant expression dimorphism after 100 generations. Meanwhile, 136
690 genes evolved for a reduction in their sexual dimorphism. Sexual dimorphism can be dynamic
691 when the underlying sex-specific fitness landscapes change over time. Selection on the
692 standing genetic variation in the sex-biased regulatory architecture would tune the gain and
693 loss of sexual dimorphism.

694

695 **Fig. 2-Fig. S1. Ovarian dormancy incidence at 12°C.**

696 Evolved females have a lower dormancy incidence than ancestral ones (Wilcoxon's test, $W =$
697 3.5 , $p = 0.061$).

698

699 **Fig. 2-Fig. S2. Time male flies attempting to copulate.**

700 Evolved males spent significantly more time chasing females (Wilcoxon's test, $W = 1174$, $p <$
701 0.001).

702

703 **Fig. 4-Fig. S1. Sex-specific responses to discordant selection via sex-biased loci.**

704 **a.** The phenotypic response of a trait controlled by 50 loci after 100 generations of sexually
705 discordant selection. Different numbers of sex-biased loci in each sex are shown. For each
706 scenario, 100 independent computer simulations were performed. The normalized phenotypic
707 change is calculated as the ratio between phenotypic change and phenotypic variance of the
708 ancestral population. **b.** Fraction of simulations in which there's a simultaneous increase in
709 male but decrease in females of the focal trait. The statistical significance denoted by "*" is
710 based on one-sample proportion test comparing to the control simulation without any sex-
711 biased locus. Bonferroni's correction is applied.

712

713 **Method-Fig. S1. Numeric example for the allometric estimation.**

714 An allometric estimate ($\hat{\alpha}_t$) measures the abundance of a gene in gonads relative to the overall
715 (mean) abundance in the whole body, reflecting the relative size of gonad in whole body. It
716 may differ between populations. Genes with different expression levels in each tissue (gene1
717 in the figure) would be affected and thus are informative for the estimation. However, for genes
718 with similar expression in different tissues (gene2 in the figure), they would be affected and
719 the estimation of $\hat{\alpha}_t$ would be meaningless.

720

721 **Method-Fig. S2. Allometric estimate of gonadal tissues in whole bodies of each gene.**

722 An allometric estimate ($\hat{\alpha}_t$) is the coefficient measuring the abundance of a gene in gonad
723 relative to the overall abundance in the whole body. The distributions of the estimates differ
724 significantly between evolved and ancestral populations in both sexes (Kolmogorov-Smirnov
725 test, $D = 0.18$ and 0.12 for females and males, respectively; $p < 0.001$ in both tests).

726

727 **Titles and legends for supplementary Files**

728 **Supplementary File 1. Likelihood ratio test for different contrasts.**

729 The file records the design matrix and results of likelihood ratio test for three different contrasts
730 between (1) evolved and ancestral samples in males, (2) evolved and ancestral samples in
731 females and (3) male and female samples.

732

733 **Supplementary File 2. Gene ontology (GO) enrichment analysis on genes of interest.**

734 Results of gene ontology enrichment analysis using topGO among different sets of genes
735 showing distinct expression changes were shown.

736

737 **Supplementary File 3. Enrichment analysis of genes highly expressed in each tissue
738 among the genes of interest.**

739 In this file, we reported the results of Fisher's exact test for enrichment of genes highly
740 expressed in each tissue among the genes of interest and the list of genes that are highly
741 expressed in each tissue.

742

743 **Supplementary File 4. All expressed TFs annotated by RcisTarget and their evolutionary
744 patterns.**

745 The genomic position, sex-specific evolutionary pattern and gene description of all expressed
746 transcription factors (TFs) annotated by RcisTarget are shown.

747

748 **Supplementary File 5. Enrichment of cis-regulatory elements and identification of**

749 **putative TFs among genes of interest**

750 The outputs of RcisTarget that test for enrichment of cis-regulatory elements among each set
751 of genes of interest are shown. The identities, expression patterns, and functional descriptions
752 of the TFs that putatively regulates genes of interest are summarized.

753

754 **Supplementary File 6. TFs that regulate sex-biased expression, expression evolution and**
755 **showed significant evolution in their expression.**

756 A list of candidate TFs that satisfy the three criteria supporting the hypothesis that selection on
757 sex-biased transcription factors may facilitate rapid sex-specific evolution in gene expression.

758

759 **Supplementary File 7. Mapping statistics.**

760 Mapping statistics of all the samples involved in the tests in this study are reported.

761

762 **Supplementary File 8. Robustness of the estimation of allometric coefficients (α).**

763 The Robustness of the estimation was evaluated with Jackknife sampling. The correlation of
764 the estimates between each pair of Jackknife samples are reported.

765

766 **Supplementary File 9. Prediction accuracy of the whole body expression using the**
767 **estimated allometric coefficients (α) and the expression profiles in dissected samples.**

768 For each Jackknife sampling, the estimated allometric coefficients (α) were applied to predict
769 the whole body expression of the left-out sample based on its expression profiles in gonad and
770 carcass. Pearson's correlation between the true and predicted values were reported.