Masculinization of the X-chromosome in aphid soma and gonads

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Summary

Males and females share essentially the same genome but differ in their optimal values for many phenotypic traits, which can result in intra-locus conflict between the sexes. Aphids display XX/X0 sex chromosomes and combine unusual X chromosome inheritance with cyclical parthenogenesis. Theoretical and empirical works support the hypothesis that the large excess of male-biased genes observed on the aphid X chromosome compared to autosomes evolved in response to sexual conflicts, by restricting the products of sexually antagonistic alleles to the sex they benefits. However, whether such masculinization of the X affects all tissues (as expected if it evolved in response to sexual conflicts) or is limited to specific tissues remains an open question. Here, we measured gene expression in three different somatic and gonadic tissues of males, sexual females and parthenogenetic females of the pea aphid. We observed a masculinization of the X in each of the studied tissues, with male-biased genes being 2.5 to 3.5 more frequent on the X than expected. We also tested the hypothesis that gene duplication can facilitate the attenuation of conflicts by allowing gene copies to neo- or sub-functionalize and reach sex-specific optima. As predicted, X-linked copies of duplicated genes having their other copies on autosomes were more frequently male-biased (40.5% of the genes) than duplicated autosomal genes (6.6%) or X-linked single-copy genes (32.5%). These results highlight a peculiar pattern of expression of X-linked genes in aphids at the tissue level and provide further support for sex-biased expression as a mechanism to attenuate intra-locus sexual conflicts.

Introduction

Sexual dimorphism, the difference between males and females at any phenotypic trait such as behavior, morphology, physiology or life history, is widespread. These differences are pervasive among eukaryotes, from plants to nematodes, insects, birds and mammals, to name a few (Cox and Calsbeek 2009; Williams and Carroll 2009). Regardless of its extent, sexual dimorphism engages males and females in a constant tug-of-war because their reproductive interests (such as optimal mating rate, number of partners, parental investment…) never align, owing to constitutive investment differences in gametes and/or progeny (Bonduriansky and Chenoweth 2009).

Differences in optimal trait values between sexes may generate intra-locus sexual conflicts. Typically, a new allelic variant could be beneficial to a female but deleterious to a male or vice versa. Such a sexually antagonistic (SA) allele is predicted to increase in frequency as long as the cost/benefit balance is positive. This increase leads to a so-called gender load in the population, due to the transmission of SA alleles to both sons and daughters (Chippindale et al. 2001; Rice and Chippindale 2002; Bonduriansky and Chenoweth 2009).

Several mechanisms may alleviate gender load (Bonduriansky and Chenoweth 2009). One is the evolution of sex-biased or sex-specific gene expression through a modifier of expression (Rice 1984). Once a SA allele is frequent enough, the reduction of its expression in the sex where it is deleterious may allow this variant to further increase in frequency and to possibly reach fixation (Rice 1984; Ellegren and Parsch 2007; Bonduriansky and Chenoweth 2009). This implies that the reduction of expression of the SA allele is beneficial to individuals of this sex. For genes that must be expressed at a certain level, a gene duplication event could allow bringing a new gene copy to sub- or neo-functionalize toward the sex-specific optimum (Bonduriansky and Chenoweth 2009; Connallon and Clark 2011; Gallach and Betrán 2011). Interestingly, these two processes (the duplication and the change in expression) could occur simultaneously, when the duplicated copy inserts in a region of the genome that already shows specific expression pattern (e.g., Arthur et al. 2014).

69 The invasion of the population by a SA allele and the attenuation of gender load
70 through duplication and/or evolution of sex-biased gene expression may take place at
71 different timescales. Indeed, the increase in frequency of a SA allele can be as rapid as a few
72 generations, depending on its effect on fitness (e.g., Dean et al. 2012 for an experimental
73 demonstration). The attenuation of the conflict by expression change or gene duplication
74 may take much longer as it relies on rare random events, themselves depending on effective
75 population size and mutation rate (Rice 1984; Stewart et al. 2010; Connallon and Clark 2011;
76 Collet et al. 2016).

77 Importantly, the conditions for invasion by a SA allele differ between autosomes and
78 sex chromosomes (Rice 1984; Fry 2010). In XX/XY systems, any SA allele that benefits males
79 can invade the Y without conflict assuming complete linkage between the SDR (sex-
80 determining region) and the SA locus. The picture for the X is more complex (Vicoso and
81 Charlesworth 2006). X-linked recessive alleles are exposed to selection in males, while the
82 female-biased transmission of the X (X chromosomes are transmitted twice more often by
83 females than by males) gives more importance to selection episodes occurring in females.
84 As a result, the X should accumulate recessive male-beneficial alleles and dominant female-
85 beneficial ones. Similar processes are expected to occur in ZZ/ZW systems (e.g., birds,
86 lepidopterans…).

87 Aphids constitute an interesting model to study the evolution of SA alleles as they
88 show an XX/X0 sex-determining system combined with cyclical parthenogenesis: the
89 alternation between several parthenogenetic generations in spring and summer and a single
90 sexual generation in autumn. As a result, three distinct reproductive morphs occur in
91 aphids: males, sexual females and parthenogenetic (asexual) females. Sexual females are
92 genetically identical to their parthenogenetic mother, while male production involves the
93 random elimination of one of the X (Wilson et al. 1997). Furthermore, during
94 spermatogenesis only sperm cells carrying an X chromosome develop (Blackman 1987), so
95 that the fusion of a sperm cell (AX) and an ovum (AX) always produces a diploid individual at
96 the X and autosomes, which develops into a parthenogenetic female.

97 Theoretical models (Jaquiéry et al. 2013) predict that the peculiar inheritance of the
98 X in aphids, the alternation between sexual and asexual reproduction, and the presence of

99 three different morphs (sexual females, parthenogenetic females and males) have a major
100 influence on the genomic location of SA allelic variants. In particular, conditions for the
101 invasion of variants that are beneficial to males and deleterious to parthenogenetic females
102 are predicted to be less restrictive for the X than for autosomes. By contrast, the conditions
103 for the invasion of variants that are detrimental to males and beneficial to parthenogenetic
104 females are more restrictive for the X. These models thus predict the X to be optimized for
105 male functions. Genomic analyses on the pea aphid *Acyrthosiphon pisum* showed that the X
106 chromosome had a large excess of genes preferentially expressed in males (i.e., male-biased
107 genes) compared to autosomes, and a deficit of parthenogenetic female-biased genes,
108 resulting in a "masculinization" of this chromosome (Jaquiéry et al. 2013). This pattern
109 matched predictions made under the hypothesis that evolution of sex-biased gene
110 expression reduces sexual conflicts by decreasing the expression of a sexually antagonistic
111 allele to the sex it benefits (Rice 1984). Interestingly, masculinization of the X has also been
112 observed in another aphid species (*Myzus persicae*) that diverged from the pea aphid
113 lineage 40 MYA (million years ago) (Mathers et al. 2019), but not in psyllids (Li et al. 2020) –
114 a closely related group to aphids but which undergoes obligate sexual reproduction. These
115 studies provide further support that the masculinization of the X evolved in response to
116 intra-locus sexual conflicts resulting from the peculiar life cycle (cyclical parthenogenesis)
117 and X inheritance in aphids. However, as previous studies on aphids analyzed whole-body
118 transcriptomes (Jaquiéry et al. 2013; Jaquiéry et al. 2018; Mathers et al. 2019; Li et al. 2020),
119 it is unknown whether the observed masculinization of the X systematically occurs within
120 each type of tissue or is driven by some specific tissue with unusual expression patterns.
121 Indeed, empirical studies in model species revealed considerable variation between tissues.
122 For example, other factors such as meiotic-sex chromosome inactivation (MSCI) makes sex-
123 chromosomes an inappropriate location for spermatogenesis genes in *Drosophila* and
124 mammals (e.g. Khil et al. 2004, Vibranovski et al. 2009). Dosage compensation may also
125 differ between tissues (Nozawa et al. 2013, Vensko and Stone 2015), which would explain
126 why the *Drosophila* X is enriched in male-biased genes expressed in the brain, but shows no
127 excess or even a deficit of male-biased genes for all other tissues (Huylmans et al. 2015).
128 Given the scarce knowledge of dosage compensation and MSCI in aphids (the only four
129 studies that analyzed gene expression in aphid males were performed on whole individuals,

130 Jaquiéry et al. 2013, Mathers et al. 2019, Liu et al. 2021, Ziabari et al. 2022), we were not
131 able to account for these factors in our theoretical predictions. Thus, we focused primarily
132 on testing predictions assuming sexual antagonism, but explored the presence of dosage
133 compensation (and MSCI to a lesser extent) on the tissue-specific transcriptomes collected
134 for this study.

135 Here, we predicted that if intra-locus sexual conflict is a strong driver of the
136 masculinization of the aphid X chromosome, masculinization would occur in all tissues. To
137 verify this prediction, we measured gene expression in different tissues from males, sexual
138 females and parthenogenetic females, including gonadic and somatic tissues. Sex-biased
139 genes were more frequent in gonads than in less sexually dimorphic tissues, nevertheless
140 we observed a masculinization of the X in each type of tissue, suggesting that - in each
141 tissue - male-beneficial alleles were favored on the X and that intra-locus conflict may be
142 resolved through the evolution of sex-biased gene expression. Moreover, we confirmed that
143 the X-linked copy of a duplicated gene having another copy on autosomes is more likely to
144 show a male-biased expression than its autosomal copy or an X-linked single-copy gene. This
145 result suggests that duplications facilitate sub- or neo-functionalization toward the sex-
146 specific optimum.

147

148 # Results

149 ## *Gene expression levels*

150 Gene expression levels in three different tissues (heads, legs and gonads) of the three
151 morphs (males, sexual females and parthenogenetic females) were measured from RNA-seq
152 counts on individuals produced by the same pea aphid clonal lineage (Supplementary table
153 S1). Overall, 14,605 genes out of the 20,639 predicted genes were expressed (> 1 count per
154 million reads [CPM] in at least two samples) in the 18 samples (3 morphs × 3 tissues × 2
155 replicates). We assigned 18,719 (90.7%) of the 20,639 predicted genes as autosomal or X-
156 linked, based on scaffold assignments from Jaquiéry et al. (2018). The genes that were not
157 assigned (9.3%) were located on scaffolds or part of scaffolds that were not clearly assigned
158 to X or autosomes in Jaquiéry et al. (2018) and they were thus not considered in the

159 subsequent analyses. Only 51% (3044/5961) of the X-linked genes were found to be
160 expressed, against 85% (10,890/12,758) of the autosomal genes (figure 1A). The genes
161 identified as not expressed in the 18 samples also generally showed no or low expression in
162 whole-body RNAseq of males and females (Jaquiéry et al. 2013), especially for X-linked
163 genes (see supplementary text S1 for details). On average, more genes were expressed in
164 the samples from the different male tissues than in female samples, especially for X-linked
165 genes (X-chromosome: median number of expressed genes in males = 2020, median in
166 females = 1425, two-sided Mann-Whitney test, $p = 0.0009$; autosomes: median in males =
167 9061, median in females = 8560, two-sided Mann-Whitney test, $p = 0.067$, figure 1B).

168 In the heatmap based on gene expression levels (figure 1C, supplementary figure S1),
169 samples grouped systematically by replicate of the same condition, and then by tissue for
170 leg and head samples. Within each of these tissues, the four female samples were always
171 more similar to each other than to the male samples. Gonad samples were the most
172 heterogeneous ones, samples from testes (MG) being highly different from all other
173 samples, and samples from parthenogenetic and sexual female gonads grouping together.

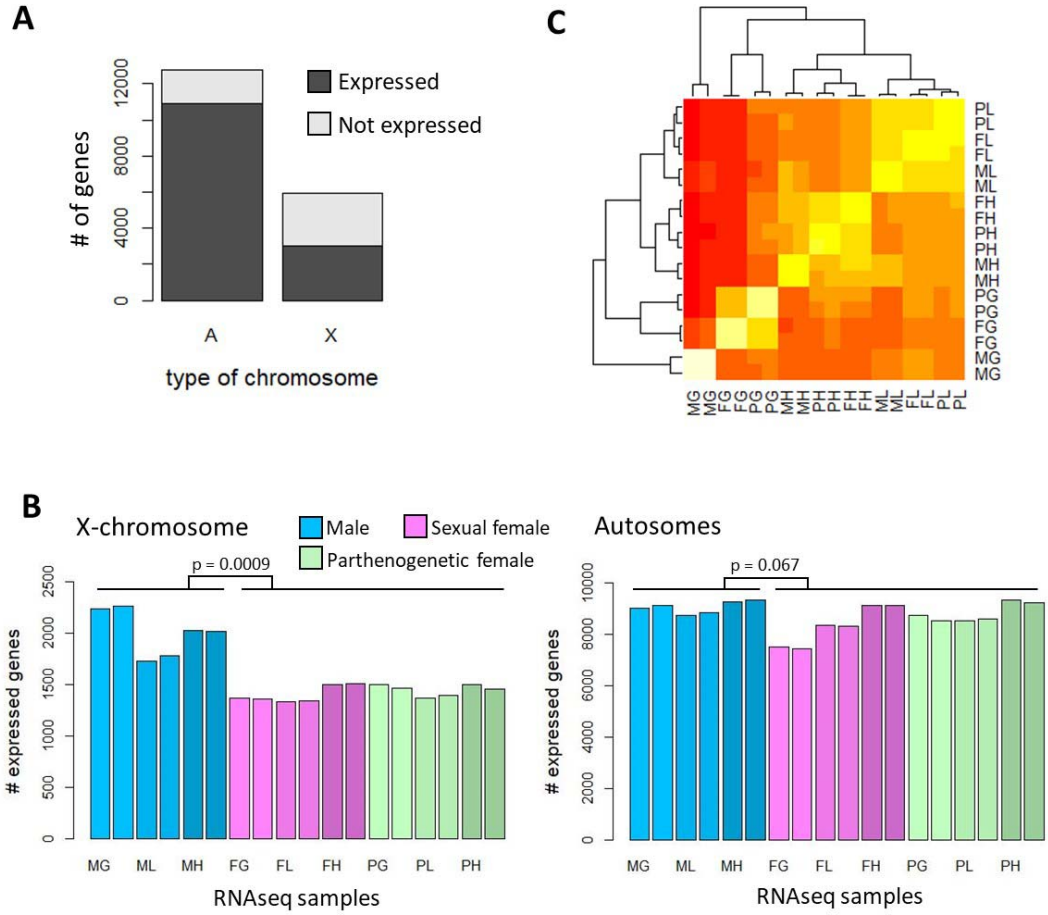


Figure 1. Gene expression in the 18 different RNA-seq samples. A) Number of genes considered as expressed (CPM>1 in at least two samples) and not expressed on the X and on the autosomes. B) Number of expressed genes per sample (expressed at more than 1 CPM) for X-linked and autosomal genes (significance of differences between the X and autosomes was estimated with two-sided Mann-Whitney tests). C) Heatmap of log(CPM+1). Samples group by tissue for head (H) and leg (L) samples, with the parthenogenetic (P) and sexual female (F) samples being always more similar compared to the male samples (M). Expression patterns of male gonad samples (MG) are the most divergent. MG: male gonad, MH: male head, ML: male leg, FG: sexual female gonad, FH: sexual female head, FL: sexual female leg, PG: parthenogenetic female gonad, PH: parthenogenetic female head, PL: parthenogenetic female leg.

The X chromosome is enriched in male-biased genes at the tissue-level

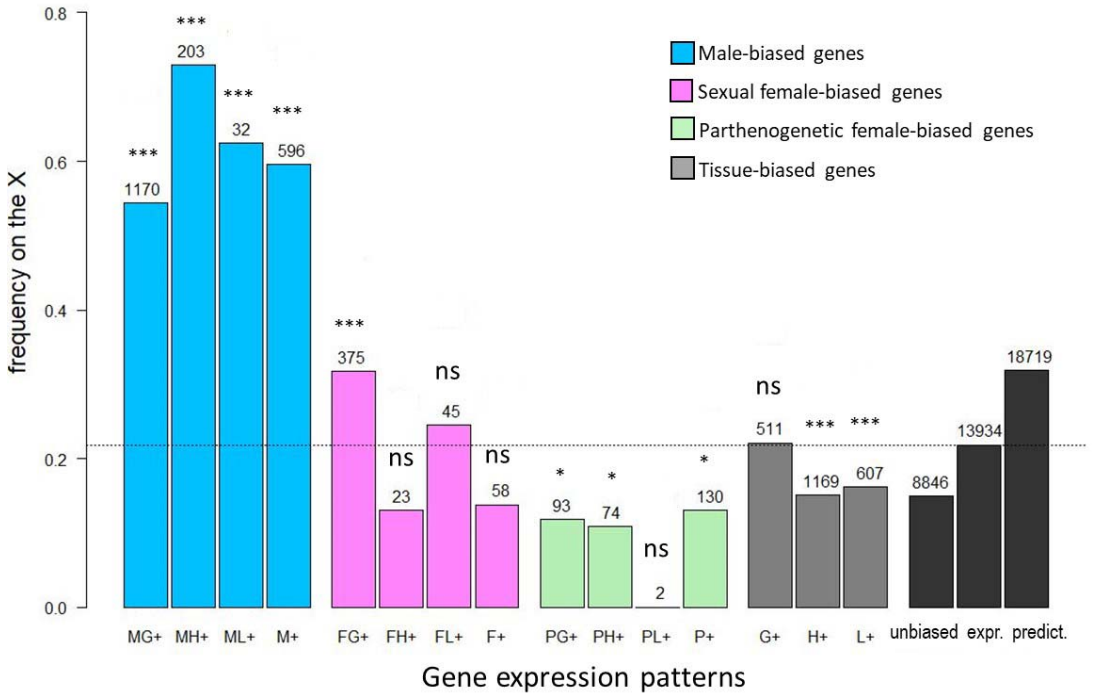
To test whether some masculinization of the X was observed at the tissue-level, we first categorized genes according to their relative expression patterns in the different conditions.

188 We defined a gene as "biased" toward, or preferentially expressed in, a set of samples
189 (which can be a particular tissue from a particular morph, all the tissues from a particular
190 morph or a particular tissue in all morphs) when at least 70% of all reads mapping to this
191 gene were observed in this set of samples (see methods). Note that increasing this
192 threshold to 80% or 90% or decreasing it to 60% or 50% did not qualitatively change the
193 results (Supplementary Figure S2). Testes showed the highest number of biased genes, with
194 1170 genes (referred to as MG+ genes) being preferentially expressed in this tissue. Then
195 came sexual female ovaries, with 375 FG+ genes, and male heads, with 203 MH+ genes
196 (figure 2). Overall, the number of sex-biased genes was higher in highly sexually dimorphic
197 tissues (gonads) than in less sexually dimorphic tissues (heads and legs). Tissue-biased genes
198 (i.e., genes expressed mainly in a tissue of all morphs) were common. Heads showed the
199 highest number of tissue-biased genes (1169 H+ genes), followed by legs (607 L+ genes) and
200 gonads (511 G+ genes). Contrastingly, morph-biased genes (i.e., genes expressed mainly in a
201 morph in all tissues) were much less frequent for females (only 58 F+ and 130 P+ genes)
202 than for males (596 M+ genes) (figure 2). When considering all genes preferentially
203 expressed in a given morph, without considering tissues (e.g., by summing MG+, MH+, ML+
204 and M+ genes for males), a total of 2001, 501 and 299 genes were biased toward males,
205 sexual females and parthenogenetic females, respectively.

206 Interestingly, these different categories of genes differed in their chromosomal
207 locations (figure 2, Supplementary table S2). The proportions of X-linked genes among
208 genes expressed preferentially in testes (MG+), male heads (MH+), male legs (ML+) or
209 simply in males regardless of tissue (M+) varied from 54% to 73%, and significantly
210 exceeded (two-sided binomial tests, $p < 10^{-7}$ in all cases) the null expectation, which we took
211 as the proportion of X-linked genes among all expressed genes (22%) (if we consider all
212 predicted genes – supported by expression data or not – 31.8% locate on the X).
213 Contrastingly, genes that were preferentially expressed in parthenogenetic females were
214 less likely to locate on the X than expected (two-sided binomial tests, p ranging from 0.014
215 to 0.023 for PG+, PH+ and P+, not significant for PL+), with proportions of X-linked genes
216 ranging from 0% to 13% depending on tissues. The proportion of X-linked genes among
217 sexual female-biased genes were intermediate (13% to 32%), with only those preferentially
218 expressed in sexual female gonads being more frequent on the X (two-sided binomial test, p

219 = 10^{-5}). Genes that were preferentially expressed in gonads (G+) showed no deviation from
220 the null expectation, as 22% of them located on the X, while genes preferentially expressed
221 in heads (H+) and in legs (L+) were significantly less frequent on the X (15% to 16%) than
222 expected (two-sided binomial tests, $p = 10^{-8}$ and $p = 0.0006$, respectively). Baring the strong
223 difference between the X and autosomes, the distribution of biased genes within
224 chromosomes was rather homogeneous (Supplementary Figure S3).

225 We found that the breadth of gene expression, measured with τ (an index that
226 ranges from of 0 – indicating similar expression in all conditions – to 1 – indicating
227 expression in one condition only) was significantly narrower for X-linked genes than for
228 those on autosomes (median $\tau_X = 0.71$, $\tau_A = 0.33$, Mann-Whitney U-test, U = 23,201,000, $p <$
229 10^{-15}).



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231 **Figure 2.** Proportions of X-linked genes among genes preferentially expressed in various morphs
232 and/or tissues. Blue bars represent male-biased genes; MG+, MH+ and ML+: genes expressed
233 preferentially in male gonads, heads and legs, respectively. M+: genes preferentially expressed in
234 males when pooling all tissues, excluding the genes assigned to the previous categories. Pink bars
235 represent sexual female-biased genes, with F standing for females and letters G, H and L having the
236 same meaning as in males. Green bars represent parthenogenetic female-biased genes (P). Grey

237 bars represent genes expressed preferentially in one of the tissues (gonads, heads or legs) and not
238 limited to a particular morph. Black bars represent the frequency of X-linked genes among genes
239 with unbiased expression ("unbiased"), genes expressed with CPM > 1 in at least two libraries
240 ("expr.") or all predicted genes ("predict."). The horizontal dotted line represents the proportion of
241 X-linked gene among expressed genes. The number of genes from each category is shown above
242 bars, as well as the p-value (two-sided binomial tests against the expected frequency on the X
243 chromosome estimated from expressed genes, which corresponds to the dotted horizontal black
244 line). ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: $p \geq 0.05$.

245 *Expression patterns of single- and two-copy genes*

246 To investigate the extent to which gene duplication facilitates the evolution of gene
247 expression toward the sex-specific optimum, we compared the expression of autosomal and
248 X-linked genes that belong to single-copy and multicopy gene families. Multigenic gene
249 families were identified by Boulain et al. (2018) from orthoDB on 17 arthropod genomes.

250 We found that the X chromosome contained more genes that belong to multicopy
251 families than autosomes (38% of the genes on the X belong to multicopy families, against
252 28% for autosomal genes). When restricting our analyses to genes supported by expression
253 data, 1633 and 7428 single-copy genes locate on the X and on autosomes, respectively. We
254 also found 210 gene families composed of two expressed genes with one being on the X and
255 the other on autosomes. On autosomes, the percentages of genes with male-biased
256 expression (combining M+, MG+, ML+ and MH+ genes) were very similar between single-
257 and two-copy genes, at 6.5% and 6.6% respectively (figure 3, Chi-squared test, $\chi^2 \approx 0$, df = 1,
258 $p = 1$). On the X chromosome however, the proportion of male-biased genes was
259 significantly higher for two-copy genes (40.5%) than for single-copy genes (32.5%) (Chi-
260 squared test, $\chi^2 = 5$, df = 1, $p = 0.025$), these two proportions being much higher than their
261 equivalents on autosomes (Chi-squared tests, single-copy genes: $\chi^2 = 939.5$, df = 1, $p < 10^{-15}$,
262 two-copy genes: $\chi^2 = 64.8$, df = 1, $p < 10^{-15}$). Sexual female- and parthenogenetic female-
263 biased genes accounted only for a few percent of single- and two-copy genes. These female-
264 biased genes showed minor differences in proportion between chromosomes (significant for
265 single-copy genes only for parthenogenetic female [$\chi^2 = 4.9$, df = 1, $p = 0.027$] and for sexual
266 female [$\chi^2 = 8.9$, df = 1, $p = 0.003$], figure 3). They constituted similar proportions of the
267 single- and two-copy genes on a given chromosome type (Supplementary table S3).

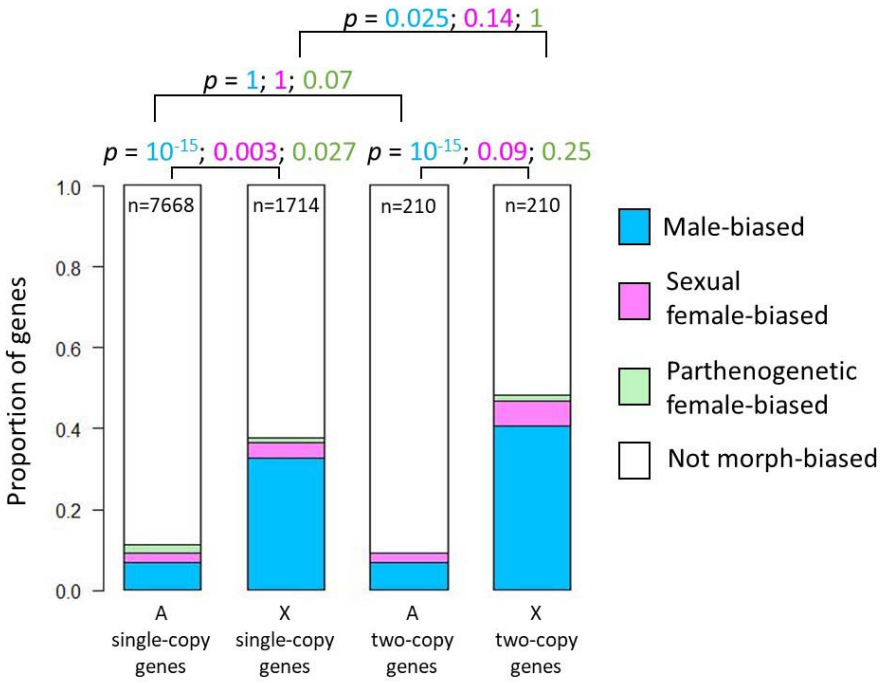
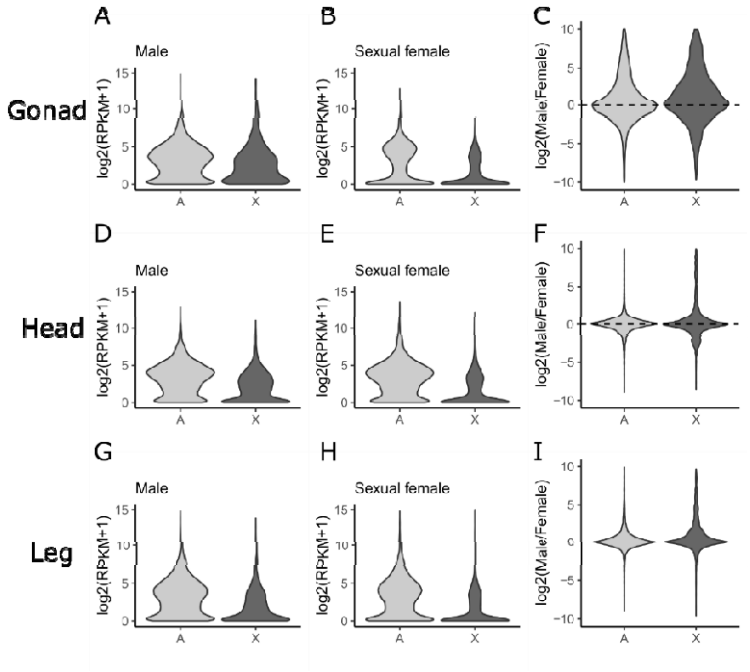


Figure 3. Proportion of genes showing preferential expression in different morphs, according to their number of copies (single or two copies) and chromosomal location (A: autosomes, X: X chromosome). Each two-copy gene has one copy on an autosome and the other on the X. P-values (Chi-squared tests) are shown, with font colors corresponding to the tested morph, according to the color of sectors. The number of genes composing each distribution is indicated on the plots.

Expression levels in morphs and tissues

The median expression levels of X-linked genes in somatic tissues and gonads from male and sexual female morphs were systematically lower than those of autosomal genes (figure 4, $p < 10^{-15}$ in all comparisons, two-sided Mann-Whitney tests), irrespective of the dose of X chromosomes per cell (two for sexual females and one for males). The same patterns were observed in parthenogenetic females ($p < 10^{-15}$ in all comparisons, supplementary figure S4). The mode of Log2 ratio of male-to-female RPKM (using sexual females in figure 4CFI and parthenogenetic females in Supplementary figure S4) lies close to 0 for both autosomal and X-linked genes, indicating dosage compensation for non-sex-biased genes in gonads and somatic tissues. Yet, we observed an excess of genes with high Log2 ratio of male to female expression, especially for the X chromosome in gonads and heads (figure 4CF). This indicates

286 an overexpression of some of the genes located on the single X chromosome of male cells,
287 which exceeds dosage compensation. This pattern was expected, given that male-biased
288 genes are significantly more frequent on the X than on autosomes (figure 2). When we
289 removed the genes characterized by a fold-change larger than 2 between males and sexual
290 females in at least one if the tissues (i.e. sex-biased genes), we still found strong evidence
291 for dosage compensation in the three tissues (Supplementary Figure S5). We also observed
292 that dosage compensation occurs throughout the X chromosome for all 3 tissues
293 (supplementary figure S6BDF), although the terminal portion of this chromosome appeared
294 to be particularly rich in sex-biased genes (supplementary figure S6ACE and S3).



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296 **Figure 4.** Logarithm of gene expression (RPKM, reads per kilobase per million mapped reads) for X-
297 linked and autosomal genes for the different types of libraries (gonads, heads or legs of males and
298 sexual females). X-linked genes are significantly less expressed than autosomal genes in all cases
299 (two-sided Mann-Whitney tests, $p < 10^{-15}$). Logarithm of male-to-sexual female ratio of RPKM is also
300 shown for the three different tissues.

301

302 ## Discussion

303 Because of the peculiar inheritance of the X chromosome in aphids – males transmit
304 systematically their unique X to all sperm cells leading to the production of female-only

305 progeny –, the presence of three distinct morphs (sexual females, parthenogenetic females
306 and males) and the alternation between sexual and asexual reproduction, a specialization of
307 the X into male functions is expected (Jaquiéry et al. 2013). Indeed, models have shown that
308 the conditions for invasion by male-beneficial/parthenogenetic female-detrimental SA
309 alleles are less restrictive for the X than for autosomes, while the opposite is true for male-
310 detrimental/parthenogenetic female-beneficial alleles. SA alleles that are favorable to
311 sexual females should show little bias, the direction of which (i.e., the depletion or
312 enrichment of the X with genes carrying such variants) depending on the selective effect of
313 the allele on males and parthenogenetic females (table 1). A key finding of these predictions
314 is that they are not qualitatively affected by allele dominance level h (the aphid X
315 chromosome is a preferred location for male-beneficial alleles for all values of $h \neq 1$). This
316 contrasts with other X0 and XY species, where the X accumulates both recessive ($h<0.5$)
317 male-beneficial alleles and dominant ($h>0.5$) female-beneficial alleles (Vicoso and
318 Charlesworth 2006; Ellegren and Parsch 2007). Consequently, simpler predictions can be
319 made on aphids: the X should be enriched with male-beneficial alleles, parthenogenetic
320 female-beneficial alleles should be more common on autosomes (being counter selected on
321 the X), and sexual-female beneficial alleles should show no consistent bias (table 1).

322 **Table 1.** Preferred chromosomal location (X chromosome *versus* autosomes) of different types of
323 alleles with morph-antagonistic effects (s_m, s_f and s_p stand for the selective coefficient of the new
324 variant on males, sexual females and parthenogenetic females, respectively). Predictions originate
325 from analytical and simulation models developed in Jaquiéry et al. (2013).

Fitness effect of a SA mutation	**Preferred chromosomal location in aphids**
$s_m > 0$, $s_f < 0$, $s_p < 0$	Favored if on the X
$s_m > 0$, $s_f > 0$, $s_p < 0$	Favored if on the X
$s_m < 0$, $s_f < 0$, $s_p > 0$	Disfavored if on the X
$s_m < 0$, $s_f > 0$, $s_p > 0$	Disfavored if on the X
$s_m < 0$, $s_f > 0$, $s_p < 0$	Slightly disfavored if on the X
$s_m > 0$, $s_f < 0$, $s_p > 0$	Slightly favored if on the X

326

327 Although the development of high-throughput approaches have allowed to pinpoint
328 putative SA genes (Innocenti and Morrow 2010; Lucotte et al. 2016; Ruzicka et al. 2019),
329 identifying these genes and estimating sex-specific selection and dominance coefficients are
330 challenging tasks that have been achieved in only a handful of studies (Barson et al. 2015;
331 Husby et al. 2015). However, since the evolution of a lower expression level in the sex that
332 suffers from an antagonistic allele could help resolving intra-locus sexual conflicts and allow
333 the SA allele to reach fixation (Rice 1984; Vicoso and Charlesworth 2006; Ellegren and
334 Parsch 2007), the chromosomal location of sex-biased genes can be used to indirectly test
335 predictions.

336 Here, we analyzed gene expression in different tissues of distinct morphs in the pea
337 aphid and found that the chromosomal location of morph-biased genes followed the
338 predictions made under SA models from Jaquiéry et al. (2013). In the three tissues
339 considered, male-biased genes were largely overrepresented on the X, parthenogenetic
340 female-biased genes were underrepresented, and sexual female-biased genes showed no
341 consistent bias. These empirical data thus support the hypothesis that, in aphids, sexual
342 conflicts would be the key driver of the masculinization of the X and of the specialization of
343 autosomes for the parthenogenetic phase of the life cycle.

344 Genes showing a male-biased expression were much more frequent on the X
345 chromosome than expected under a random distribution, irrespective of the tissue
346 considered. Genes expressed mainly in testes, male heads and male legs were 2.5 to 3.5
347 times more frequent than expected on the X, so were genes that were male-biased in all the
348 tissues considered. As all the investigated tissues contribute to this pattern, these results
349 extend previous studies reporting a general enrichment in male-biased genes (measured
350 from whole-body samples) on the aphid X chromosome (Jaquiéry et al. 2013; Jaquiéry et al.
351 2018; Mathers et al. 2019). Overall, this and previous studies demonstrate strong and
352 consistent bias toward the X in aphids for genes expressed predominantly in males.
353 Interestingly, on the X chromosome, the percentage of male-biased genes was significantly
354 higher for two-copy genes (40.5%) than for single-copy genes (32.5%), while these
355 percentages were similar for autosomal genes (6.5% and 6.6%). These results match the
356 predictions that duplications may facilitate sub- or neo-functionalization toward sex-specific
357 optima, and that, for genes located on the X chromosome, the male optimal expression
358 levels are favored rather than the female optima.

359 Conversely, parthenogenetic female-biased genes were significantly less frequent on
360 the X, except for parthenogenetic female leg-biased (PL+) genes due to lack of power, as
361 only two such genes were found, both located on autosomes. This matches previous
362 observations on whole body transcriptomes (Jaquiéry et al. 2013; Mathers et al. 2019). This
363 consistent pattern therefore reveals a reduction of the contribution of the X chromosome to
364 biological functions and processes occurring in parthenogenetic females, and corroborates
365 the observation that the X is depleted from functionally important genes for the
366 parthenogenetic phase (Li et al. 2020). Interestingly, the chromosomal location of sexual
367 female-biased genes did not significantly depart from random expectations, except for
368 genes specifically expressed in ovaries. These were significantly more frequent than
369 expected on the X, although their proportion (32%, Figure 2) was much lower than for male-
370 biased genes (54%-73%). According to the models (table 1), variants of X-linked genes that
371 are beneficial to sexual females should increase in frequency only if they are also beneficial
372 to males. This could be the case for genes controlling sexual reproduction-related functions
373 that were not sex-specific (e.g., meiosis). In this case however, we do not expect such genes
374 to be preferentially expressed in sexual females. These genes may be less/not expressed in

375 adult males because spermatogenesis is often already completed at this stage (Wieczorek et
376 al. 2019). Alternatively, it is possible that these genes had variants that were beneficial to
377 both sexes, which have since evolved functions that are more specific to sexual females.

378 Among hemipterans, the accumulation of male-biased genes on the X appears to be
379 specific of aphids. A deficit of male-biased genes on the X was observed in three hemipteran
380 species (two heteropteran bugs and a leafhopper) that only reproduce sexually and are very
381 distantly related to aphids, despite an apparent homologous origin of the X chromosome
382 (Pal and Vicoso 2015; Mathers et al. 2021). In psyllids, which diverged more recently (~200-
383 250 MYA) from aphids and are characterized by obligate sexual reproduction and X0 sex
384 determination, no enrichment of male-biased genes on the X chromosome was found (Li et
385 al. 2020). Hence, the accumulation of male-biased genes on the X chromosome in aphids
386 would have started after the divergence between aphids and psyllids. This scenario supports
387 a role for cyclical parthenogenesis (which appeared ~200 MYA in aphid ancestors, Davis
388 2012) in the masculinization of the X in aphids.

389 While the chromosomal location of the different types of sex-biased genes matches
390 the predicted evolution of gene expression as a mitigation mechanism of sexual conflicts,
391 other processes could contribute to the observed patterns. Genes located on different
392 chromosomes types could differ in other characteristics that could result in a non-random
393 genomic distribution of male-biased genes. For example, the X chromosome of *Drosophila* is
394 enriched in young genes (those that are present in only a restricted taxonomic group),
395 which are more likely to show sex-biased expression (Palmieri et al. 2014). In our analysis
396 however, the X-linked copy of a two-copy gene had a much greater probability of being
397 male-biased (0.405) than its autosomal counterpart (0.066, figure 3), demonstrating that the
398 masculinization of the X does not solely reflect differences in gene characteristics.
399 Moreover, the three *A. pisum* autosomes are systematically depleted in male-biased genes
400 and enriched in parthenogenetic female-biased genes (supplementary table S4,
401 supplementary figure S3). This reinforces the specific hemizygosity of the X in males as a
402 determinant factor for the observed patterns. Consequently, we do not see an alternative
403 hypothesis to explain the accumulation of male-biased genes on the X to the one based on
404 the resolution of sexual conflicts by an evolution of sex-specific or sex-biased gene
405 expression.

406 The number of morph-biased genes was highest for gonadic tissues, with 1170, 375
407 and 130 genes being specific of gonads of males, sexual females or parthenogenetic
408 females, respectively. This observation likely reflects the highly morph-specific functions of
409 gonads: the production of sperm in males, of yolky eggs in sexual females and of embryos in
410 viviparous parthenogenetic females (Michalik et al. 2013). In other species, testis also stands
411 out as the tissue showing the most specific gene expression patterns (Meiklejohn and
412 Presgraves 2012; Uhlén et al. 2015). Many genes also showed preferential expression in
413 male heads (202), against 23 and 93 for sexual and parthenogenetic female heads,
414 respectively. This could reflect sensorial and/or behavioral differences between males and
415 females: males have to actively search for females and initiate mating, while females spend
416 most of their time in feeding. Legs showed a low number of morph-biased genes, most of
417 which were overexpressed in sexual females (45 genes, against 32 for males and 2 for
418 parthenogenetic females). This result may reflect the existence of specific organs on sexual
419 female tibias (scent plaques), which are responsible for the secretion and release of sex
420 pheromones (Murano et al. 2018). Overall, the degree of sex-biased expression varied with
421 the degree of specialization of each tissue in sexual functions. Whether this suggests that
422 sexual antagonism is stronger in such highly sexually dimorphic tissue than in tissues with
423 more similar function in both sexes remains an open question, as causal demonstration of
424 the link between sex-biased expression and sex-specific fitness is challenging (Mank 2017).

425 Interestingly, the strong enrichment of the X with male-biased genes is similar across
426 tissues (ranging from 54% to 73%). This result strikingly contrasts with *Drosophila*, where
427 male-biased genes from different tissues show opposing patterns: male-biased genes in
428 brains are strongly enriched on the X, while there is either no departure from random
429 expectation or a paucity of male-biased genes on the X for the other tissues (Huylmans and
430 Parsch 2015). These differences were interpreted as resulting from the interplay between
431 dosage compensation (the brain could be more sensible to gene dose, and thus would
432 require a tighter dosage control) and sex-specific regulation of gene expression (Huylmans
433 and Parsch 2015). In aphids, the consistent enrichment of the X with male-biased genes in
434 different tissues suggests that similar evolutionary forces apply to solve SA conflicts in
435 reproductive and somatic tissues. Under the hypothesis that sex-biased expression evolved
436 in response to sexual conflicts, our results suggest that their attenuation occurs in all

437 tissues. Our data also indicate that - even if sexual conflicts could be more frequent in
438 gonads (assuming that sex-biased genes reveal past or ongoing conflicts) - conflicts also
439 occur in a wide range of somatic tissues. This pattern was also observed by Innocenti and
440 Morrow (2010), who found that transcripts showing signature of sexual antagonism in
441 *Drosophila* were frequent in soma.

442 The high expression of a substantial number of X-linked genes in males despite the
443 haploid state of this chromosome in this sex is intriguing. The X chromosome shows several
444 specific characteristics, among which a slightly larger amount of intra-chromosome
445 duplicates (449 for 132 Mb) compared to the largest autosomes (413 for 170 Mb, Li et al.
446 2019), and an enrichment with multi-copy orthologs (Li et al. 2020). Our analyses confirmed
447 this trend, as 38% of the genes on the X belongs to multicopy families, against 28% for
448 autosomal genes. It is yet to be determined whether recent (undetected) duplications are
449 more common on X and could contribute to high expression of some of the X-linked genes
450 in males. However, epigenetic mechanisms probably play a more important role. Indeed,
451 the X-chromosome is hypermethylated in male aphids compared to autosomes, and
452 differential gene methylation between males and females positively correlates with
453 differential expression, especially for the X chromosome (Mathers et al. 2019). An increased
454 accessibility of the chromatin of the X in males was also documented (Richard et al. 2017).
455 Another interesting feature of the aphid X chromosome is its larger fraction of
456 unexpressed genes compared to autosomes, amounting to half of the X-linked genes based
457 on our tissue samples data (against 15% of the autosomal genes). This characteristic was
458 also underlined from whole body transcriptomes (Jaquiéry et al. 2013; 2018; Richard et al.
459 2017; Li et al. 2020; Mathers et al. 2019). Two factors may contribute to the large number of
460 X-linked genes classified as unexpressed. X-linked genes might show a narrower expression
461 breadth, being restricted to some (unsampled) morphs, tissues or stages. The exceptionally
462 high τ value for expressed X-linked genes indeed provides some support to this hypothesis.
463 Nevertheless, a large fraction of the X-linked genes classified as "unexpressed" here also
464 shows low expression support in whole body libraries (see supplementary text S1): 40% are
465 supported by 0 reads and 34% by 1-5 reads. It is thus probable that both effects (absence of
466 expression or narrower expression breadth) explain the large fraction of unexpressed X-
467 linked genes. RNA sequencing on a larger diversity of tissues (especially in males) and stages

468 may be required to resolve this point. Interestingly, Li et al. (2020) found that genes
469 considered as "functionally important" were less likely to locate on the X chromosome. The
470 evolutionary forces that drive these patterns remain to be identified, but they could be
471 linked to sexual antagonism or to the particular epigenetic state of the X.
472 Previous studies suggested dosage compensation in the pea aphid and the green
473 peach aphid from whole-body transcriptomes (Jaquiéry et al. 2013; Pal and Vicoso 2015;
474 Richard et al. 2017; Li et al. 2020; Mathers et al. 2019). Here, we show dosage compensation
475 in all investigated tissues, including testis. While single-cell transcriptomics will be essential
476 to demonstrate or refute that X-linked genes are dosage compensated in various cell types
477 during spermatogenesis, dosage compensation in testis would be another peculiarity of
478 aphids (but see Witt et al. 2021; Mahadevaraju et al. 2021). Indeed, sex chromosomes of
479 other dosage-compensated species seem generally not compensated in the gonads of the
480 heterogametic sex in diptera and lepidoptera, at the scale of entire organs (Vicoso and
481 Bachtrog 2015; Gu et al. 2017; Gu and Walters 2017). However, single-cell RNAseq analysis
482 of *Drosophila* testis has evidenced dosage compensation in pre-meiotic and somatic testis
483 cells (Witt et al. 2021, Mahadevaraju et al. 2021). In other groups (mammals, birds,
484 nematods, fungi), sex-linked genes are silenced by meiotic sex chromosome inactivation or
485 MSCI (Shiu et al. 2001; Bean et al. 2004; Turner 2007; Schoenmakers et al. 2009; but see
486 Guioli et al. 2012; Daish et al. 2015), and recent studies suggest that MSCI could also occur
487 in *Drosophila* (Mahadevaraju et al. 2021; Witt et al. 2021). Different hypotheses have been
488 proposed to explain the silencing of sex-linked genes during gametogenesis. MSCI could be a
489 consequence of the mechanisms that protect against unwanted recombination between X
490 and Y and allow DNA repair in its absence (Lu et al. 2015). MSCI may also has evolved as a
491 mean to protect unsynapsed chromosomes from the invasion of transposons (Huynh et al.
492 2005) or segregation distorters that would bias sex-ratio (Meiklejohn and Tao 2010). If
493 protection from segregation distorters is an important driver of sex-linked gene silencing,
494 this could explain why the expression of X-linked genes in aphid testis has not been
495 repressed during evolution and thus can be subject to dosage compensation. In aphids,
496 segregation distortion is already maximal (all sperm cells that do not carry an X degenerate,
497 and those that are functional carry the single identical X chromosome), so no X-linked allele
498 can further increase its transmission during spermatogenesis. Hence, there is no possibility

499 for X-linked distorters to evolve, such that mechanisms to protect from distorters (i.e., sex-
500 linked gene silencing) may have been lost.
501 In conclusion, we document an atypical genome-wide pattern of gene expression in
502 aphids, with a high degree of masculinization of the X chromosome in both somatic and
503 gonadic tissues. Our study reinforces the hypothesis that this masculinization evolved in
504 response to sexual conflicts raised by the accumulation of male-beneficial alleles on the X
505 (Jaquiéry et al. 2013). To further support this hypothesis, masculinization of the X should be
506 assessed in distantly related aphid lineages (which diverged up to 200 MYA, Davis 2012) and
507 other species that show an "aphid-like" life cycle and X-inheritance (e.g. *Strongyloides*
508 nematods, Nemetschke et al. 2010; Streit 2017). Finally, understanding the functional
509 epigenetic or post-transcriptional mechanisms responsible for sex-biased gene expression in
510 aphids would help to understand how such a strong chromosomal specialization in gene
511 expression has been achieved.

512

513 Methods

514 *Sex- and tissue-biased gene expression analysis*

515 Gene expression levels in three different tissues (head, legs, gonads) of three reproductive
516 morphs (male, sexual female, parthenogenetic female) were measured from RNA-Seq
517 collected on a single pea aphid genotype (clone LSR1, from alfalfa, IAGC 2010). Aphids were
518 reared on broad bean *Vicia faba* at less than five individuals per plant to prevent the
519 production of winged morphs. Parthenogenesis was maintained under a 16-hour light
520 regime and a temperature of 18°C. The production of sexual individuals was initiated by
521 transferring larvae (at stage 3) to a 12-hour light regime at the same temperature of 18°C.
522 Two generations later, sexual females and males were observed. A total of 100 adult
523 parthenogenetic females (produced under 16-hour light regime), 100 adult sexual females
524 and 100 adult males were immediately frozen into liquid nitrogen. Heads and legs were
525 scalpel-cut. Twenty additional individuals per morph were also dissected in a saline solution
526 with fine forceps to collect gonads. Gonads included testes and accessory glands in males
527 and ovarioles in sexual females. Embryos of stage >10 (according to Miura et al. 2003) were

528 removed from parthenogenetic female ovarioles (which already contain asexually
529 developing embryos) to avoid the contribution of developing and late embryos to RNA
530 production. All collected tissues were stored in RNA later (Qiagen) immediately after
531 collection and pooled in batches before RNA extraction (with two replicates by sex and
532 tissue). Hence, a total of 18 RNA extractions (3 morphs × 3 tissues × 2 biological replicates)
533 were performed using the SV Total RNA Isolation System (Promega) according to
534 manufacturer's instructions. RNA quality was checked on Bioanalyzer (Agilent) and
535 quantified on Nanodrop (Thermo Scientific). The 18 RNA samples were subsequently sent to
536 the GetPlage platform (Toulouse, France) for library preparation (TruSeq Stranded mRNA
537 Library Preparation kit) and 150 bp RNA paired-end sequencing (Illumina HiSeq3000).

538 After filtering for rRNA, reads from each library were mapped to the V2 assembly of
539 the pea aphid genome (Acyr 2.0, Genbank accession GCA_000142985.2) using STAR version
540 2.5.2a (Dobin et al. 2013) with default parameters, except: outFilterMultimapNmax = 5,
541 outFilterMismatchNmax = 3, alignIntronMin = 10, alignIntronMax = 50000 and
542 alignMatesGapMax = 50000. Then, we counted reads mapped on exons of each predicted
543 gene (NCBI Annotation release ID: 102) using FeatureCounts version 1.5.0-p3 (Liao et al.
544 2014) with default parameters except: -g gene -C -p -M --fraction. The numbers of mapped
545 reads per library ranged from 19.8 to 28.6 million (mean 24.3 million, supplementary table
546 S1).

547 We used the R package DESeq (Anders and Huber 2010) to normalize the libraries
548 (upperquartile method with p = 0.75) and calculate CPM. Only genes with CPM>1 in at least
549 two libraries (out of 18) were considered as expressed and retained in the analyses, unless
550 mentioned otherwise. To identify genes predominantly expressed in a specific tissue or a
551 morph, we imposed that a minimal percentage (70%) of the reads mapping to a given gene
552 was sequenced from that specific tissue/morph. Doing so avoided the reliance on p-values
553 from differential expression analyses (which in turn depend on the absolute expression level
554 of a gene). This 70% threshold allowed identifying genes showing considerable bias in
555 expression toward a tissue/morph, and to retrieve a large number of genes for more
556 powerful analyses. So, when >70% of the reads mapping to a gene were from a given tissue
557 from a given morph, the gene was classified as predominantly expressed in that tissue (e.g.,
558 PL+ for those predominantly expressed in legs of parthenogenetic females; etc). When >70%

559 of the reads were from a given morph (but not restricted to a single tissue), the gene was
560 classified as morph-specific (M+, F+ or P+, for males, sexual females, or parthenogenetic
561 females, respectively). Similarly, genes mainly expressed (>70% of reads) in a tissue (but not
562 restricted to a single morph) were classified as tissue-specific (G+, H+ or L+, for gonads,
563 heads or legs, respectively). Expressed genes were thus classified into 16 mutually exclusive
564 classes: MH+, ML+, MG+, FH+, FL+, FG+, PH+, PL+, PG+, M+, F+, P+, H+, L+, G+ or unbiased.
565 Additional analyses with threshold values of 50%, 60%, 80% and 90% to identify a gene as
566 predominantly expressed in a set of samples were also conducted to verify the robustness
567 of our results to this parameter.

568 *Comparisons between chromosome types*

569 The chromosomal location (X vs autosomes) of each gene was that of Jaquiéry et al. (2018).
570 This represented the only assignments available at the start of the analyses. Since then,
571 chromosome-level genome assemblies have been produced for the pea aphid (Li et al. 2019;
572 Mathers et al. 2021). We found that our assignments to chromosomes were consistent for
573 97% to 98% of the genes located on the four mega scaffolds corresponding to the four pea
574 aphid chromosomes, depending on the assembly. We therefore considered our initial
575 assignment reliable. Furthermore, the number of NCBI predicted genes that we assigned to
576 chromosome (18719) was larger than for the Li et al. (2019) assembly (17315). For the
577 genome assembly from Mathers et al. (2021), DNA was obtained from a lineage sampled on
578 *Lathyrus*, a host plant genus that harbors a cryptic species of the pea aphid complex
579 (Peccoud et al. 2009; 2014) that is quite divergent from the LSR1 clone we used (Peccoud et
580 al. 2009). Significant deviation from a random chromosomal distribution for each class of
581 genes with a specific expression pattern was tested with two-sided binomial tests, the
582 expected proportion was computed as the proportion of expressed genes on the X. To
583 compare expression patterns between the three autosomes (supplementary table S4,
584 supplementary figure S3), we used the assembly from Li et al. (2019). Expression breadth for
585 X-linked and autosomal genes was estimated with τ (Yanai 2005) on log+1 transformed data.

586 *Investigation of gene families*

587 To identify single-copy and multicopy genes in *A. pisum* genome, we used orthologs
588 identified among 17 arthropod genomes by Boulain et al. (2018). Briefly, the longest protein
589 isoform from each arthropod species was used to run OrthoDB_soft_1.6 (Kriventseva, et al.
590 2015) and the levels of orthology were assigned by referring to the species phylogeny
591 established in Boulain et al. (2018). The groups of orthologs generated by OrthoDB were
592 then used to identify *A. pisum* unique (single-copy) or duplicated (multicopy) genes. To
593 control for possible differences in gene content between the X and autosomes, which could
594 account for different expression patterns between chromosomes, we searched for gene
595 families composed of two genes, one being on the X and the other on autosomes. To
596 statistically compare frequencies of the various classes of expression between the X and
597 autosomal copies with sufficient power given the limited sample size (n = 210 pairs of
598 genes), the gene classes with male-biased expression (i.e., M+, MG+, ML+ and MH+ genes)
599 were grouped into a single category of male-biased genes. We proceeded similarly for
600 sexual female-biased genes and parthenogenetic female-biased genes. We also created a
601 new class encompassing all genes that were not morph-biased (i.e, G+, H+, L+ and unbiased
602 genes). For each aggregated gene class, we compared its frequency among chromosomes
603 for single and duplicated copies and then among single and duplicated copies within
604 chromosomes (chi-squared tests).

605 *Dosage compensation*

606 To investigate dosage compensation, RPKM (reads per kilobase per million mapped reads)
607 were calculated with EdgeR (Robinson et al. 2010), and only genes with RPKM > 1 in at least
608 two of the 18 libraries were kept. After log-transformation of RPKM, differences in
609 expression levels between chromosomes were examined with two-sided Mann-Whitney
610 tests for each sex and each tissue. Then, the logarithm of the male to female (sexual or
611 parthenogenetic) ratio of RPKM were estimated for X and autosomes for each tissue. As the
612 uneven frequency of biased genes between chromosomes could interfere with dosage
613 compensation patterns, the same analyses were performed by eliminating genes that
614 showed a fold change in expression greater than 2 between males and females in at least
615 one tissue.

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