A selfish genetic element and its suppressor causes abnormalities to testes in a fly

Authors

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Key words

Meiotic drive, X chromosome, gonads, fertility, suppression, *Drosophila subobscura*

Abstract

Selfish genetic elements (SGEs), specifically X-chromosome meiotic drive (XCMD), create huge conflicts within a host's genome and can have profound effects on fertility. Suppressors are a common evolutionary response to XCMD to negate its costs. However, whether suppressors themselves can cause negative non-target effects remains understudied. Here, we examine whether the intragenomic conflicts created by XCMD and its suppressor affect gonad morphology in *Drosophila subobscura.* We found significant differences in testes, seminal vesicle, and accessory gland size depending on whether a male carried a non-driving X chromosome, an XCMD, and if the XCMD was suppressed. We also found the first evidence of abnormal testes development that is specifically associated with a suppressor of XCMD. Unlike other studies, our evidence suggests that XCMD in *D. subobscura* creates major abnormalities to male gonads. These abnormalities are most frequent if both XCMD and its suppressor are both present. While costs of suppression have importance in theoretical models, they have largely been ignored in empirical XCMD systems. Overall, this study highlights that genetic conflict, created by SGEs and their suppressors, is a potent evolutionary force that can have major impacts on gonad development and gametogenesis.

Introduction

Gamete production, and with this testes and ovary development, are shaped by both natural and sexual selection. For instance, egg size is strongly affected by natural selection (1,2) whereas testis size and sperm number is heavily influenced by sexual selection (3,4), with trade-offs between investment in testes and somatic function. Thus, testes are classic examples of organs that have to trade-off between sexual and natural selection (5). However, gonads are also the site of an additional set of trade-offs; the need to defend against selfish genetic elements (SGEs) (6–8).

Challenging the laws of 'fair' Mendelian inheritance, SGEs can bias their own transmission into the next generation without increasing the fitness of the carrier and often at the cost of the rest of the genome (9). For example, B chromosomes are additional to an organism's normal chromosome complement, that carry superfluous DNA (10,11). They accumulate in the ova rather than polar bodies, allowing them to selfishly accumulate in the germline (10). As a result, they are widely reported in nature and can cause phenotypic differences and reduce fertility (10,12,13). Co-evolutionary responses to intragenomic conflicts created by SGEs in gonads are likely to shape their evolution, alongside natural and sexual selection. Thus, the role of SGEs on gonad evolution should not be overlooked, and could be significant and universal because SGEs are both diverse and near ubiquitous in nature (9).

Conflicts created by SGEs can have enormous consequences on the host's fertility. For instance, *P* element dysgenesis is a property of transposable elements active in *Drosophila simulans* germlines (14). Strong evidence for the impact of SGEs in evolution comes from observations when SGEs enter a naïve host that it has not coevolved with. The *P* element in its native host is not associated with any gross defects (15). Yet when this element is horizontally transmitted into naïve hosts, it becomes associated with a number of abnormalities, including sterility and dysgenic ovaries (16). Another example is X chromosome meiotic drive (XCMD), where male carriers have their Y-sperm killed or damaged thus ensuring the transmission of only the selfish X chromosome to the

54 next generation (17). This reduces a male's fertility and sperm competitive ability (18–20). Thus, a
55 range of SGEs can negatively impact their hosts and are capable of damaging the gonads and fertility
56 of both sexes. When faced with a multitude of conflicts caused by SGEs, how do organisms and in
57 particular their gonads, respond to deal with these challenges?

58 Here we investigate how the development of the testes responds when faced with XCMD
59 and a suppressor of this system. XCMD is a type of SGE that creates huge conflicts within the testes
60 (21). XCMD manipulates the process of meiosis in males, where selfish X chromosomes kill Y-bearing
61 sperm during spermatogenesis, resulting in the overproduction of daughters (17). The transmission
62 advantage gained by XCMD can allow it to rapidly spread through populations, distorting sex ratios
63 and even leading to population extinction owing to a lack of males (22–24). Thus, testes in XCMD
64 carrying males are a good model to investigate how SGEs impact the evolution of gonads.
65 Additionally, testes are particularly vulnerable to SGEs due to the asymmetry of gamete production
66 between males and females. Eggs are large and very costly for a female to produce, so anything that
67 damages eggs will be extremely costly and very quickly selected against. On the other hand, because
68 male gametes are generally massively over-produced, damage to sperm may be more readily be
69 tolerated. In cases where males only produce a reduced amount of sperm, they can still be
70 successful, making sperm and testes a good target for the activity of SGEs.

71 In XCMD, male carriers lose up to half of all the sperm that they produce (25). Despite the
72 lack of sperm produced, drive carrying males do not often show fertility costs in single matings when
73 compared to wildtype males (18,26–29). Indeed, in stalk eyed flies, the presence of sperm-killing
74 XCMD appears to have driven the evolution of a compensatory increase in the testes size of XCMD
75 males (30–32) . This allows them to produce a similar amount of sperm to non-XCMD males. These
76 studies, that look at how the testes respond to drive, raises the question as to whether drive
77 systems in general promote changes in the testes.

78 Meiotic drive is deleterious to male function, and selection commonly favours unlinked
79 suppressor factors that restore Mendelian segregation (21,33). Suppressors, either Y-linked or
80 autosomal, must have the ability to prevent some killing of Y-bearing sperm when a driving X
81 chromosome is present (34). Once a suppressor has evolved, and as long as it has no associated
82 costs, it is expected to rapidly spread to fixation (35,36). Despite this, the impact of suppressors of
83 XCMD on the testes themselves has been rarely considered in empirical studies, despite being
84 recognised as potentially important in theoretical studies (37). Ongoing co-evolution between drive
85 and its suppressor could have different outcomes depending on how a suppressor works, whether it
86 has associated costs, and if it has non-target effects. Thus, the question remains: does the
87 emergence of suppressors negate any testes phenotype (and associated selection) produced by
88 SGEs? Alternatively, the need for testes to evolve could be enhanced if they have to deal with both
89 XCMD and suppression, which are locked in a spiralling co-evolutionary arms race (37–40).

90 In this paper, we use the XCMD system in *Drosophila subobscura* to look for any effects on
91 testes morphology. *D. subobscura* harbours an XCMD system called 'SR' (41). The strength of drive is
92 strong with SR carrying males producing broods that are 85-100% female (41,42). Given this strong
93 drive, a simple expectation is that SR should rapidly spread to fixation everywhere. However, it does
94 not. Instead, in North Africa, SR has remained at a stable frequency of approximately 20% for at least
95 50 years (41–43). Additionally, *D. subobscura* are reported to be strictly monandrous (28,44) so
96 effects of sperm competition are irrelevant in this species (18,45). We recently discovered
97 suppressors of the SR system (43) that are capable of fully restoring a normal 50:50 sex ratio to SR
98 carrying males. Despite the prediction that suppressors against drive should rapidly fix within
99 populations, this suppressor remains rare at a frequency of 1-5%. The suppressor is also found in
100 multiple locations across North Africa at these low frequencies. The lack of spread of this suppressor
101 within populations may indicate that it is associated with its own costs and creates its own burden
102 upon its host. While we refer to "the suppressor", we have not fully characterised it. Initial crosses of
103 XCMD into suppressor genotypes results in partial suppression, and several generations of

104 introgression are required to reach complete drive suppression (Supplementary figure 1). Thus, we
105 believe "the suppressor" involves multiple loci across at least two chromosomes, and the Y is not
106 involved. However, in the interests of brevity, we will refer to it as "the suppressor" here, and aim to
107 dissect the complexity in future work.

108 We determine whether testes morphology is affected when males carry the selfish SR X
109 chromosome (henceforth referred to as SR males) in comparison to standard (ST) males that do not
110 carry SR. We also examine if SR males that also carry the suppressor genes (^{Supp}SR males) can restore
111 intact testes or whether the suppressor itself can have its own influence on testes morphology. We
112 used independent SR from multiple locations in Morocco to investigate whether drive in populations
113 have evolved and responded in the same way when it is paired with suppressors that originated
114 from populations in Tunisia.

115 ## Materials and Methods

116 **Origin and maintenance of fly stocks**

117 Isolines of *Drosophila subobscura* were originally collected from Tabarka, Tunisia (36.95°N
118 8.74°E) in 2013 (46), Ras el Oud, Morocco (34.15°N 4.00°W), Demnat, Morocco (31.73°N 7.01°W)
119 and Ouzoud, Morocco (32.02°N 6.72°W). Moroccan collections took place in 2019 and all flies were
120 caught using banana, yeast and beer baited traps (47). All isolines were kept in standard *Drosophila*
121 vials on an ASG double yeast medium (10g agar, 85g sucrose, 40g yeast extract, 60g maize, 1000ml
122 H_2O, 25ml 10% nipagin) at 18°C on a 12:12 light/dark cycle. Experimental flies were generated by
123 collecting unmated flies from isolines from each population. All eclosed flies were removed from
124 populations 24 hours prior to ensure flies had not mated. Fly collection was done using CO_2
125 anaesthesia to separate flies by sex. Flies were stored in new vials on a sugar yeast medium (100g
126 sugar, 100g yeast extract, 20g agar, 1000ml H_2O, 25ml 10% nipagin, 5ml propionic acid) with no
127 more than 10 flies per vial.

128 **Isolation and introgression of SR X chromosomes**

129 We isolated an SR chromosome from a wild caught fly from each of Ras el Oud, Morocco,
130 Demnat, Morocco and Ouzoud, Morocco, where SR occurs at a frequency of approximately 20%
131 (43). SR males were initially identified though a phenotypic scan by counting their offspring sex ratio.
132 Males that produced more than 85% females were deemed SR males and were used to isolate their
133 driving X chromosomes. In order to produce the SR males (that carry an SR chromosome),
134 suppressed SR males (^{Supp}SR), that carry a suppressed version of the SR chromosome, and standard
135 (ST) males (that do not carry the SR chromosome) that were used in this investigation, we
136 introgressed both vulnerable and suppressing ST isolines onto the isolated SR chromosomes. This
137 introgression process meant we were able to produce flies that only differ genetically in carrying
138 drive (males either carried an SR X chromosome or an ST X chromosome) and either carried the
139 suppression genes or genes susceptible to drive. Preliminary experiments have shown that the

140 Moroccan isolines show no suppression against SR (43), thus these isolines were used to produce the
141 SR males that produce 100% daughters. Additionally, the ST isoline from Tunisia contains genes that
142 are capable of suppressing SR. This Tunisian ST isoline was therefore used to produce the ^{Supp}SR
143 males that produce a normal 50:50 sex ratio despite the male carrying SR (Supplementary figure 1).

144 We introgressed the SR X chromosomes onto either the vulnerable Moroccan ST isolines or the
145 suppressing Tunisian ST isoline for 14 to 17 generations (Supplementary figure 2). The introgression
146 process was done in two crosses per generation. Cross 1: females homozygous for the isolated SR X
147 chromosome ('SRSR' females) were crossed to a standard ('ST') male from the target isoline. This
148 cross produces heterozygous females ('SRST' females) that are discarded, and 'SR' males that carry
149 the SR X chromosome and the target isoline autosomes. Cross 2: simultaneously, SRSR females were
150 crossed to SR males containing the isolated SR X chromosome. This cross produces only SRSR
151 females. SR males containing the target autosomes generated from cross 1 are then crossed to the
152 SRSR females generated from cross 2 to initiate the subsequent generation. ST males are also re-
153 crossed to the SRSR females from cross 2 to reset cross 1 for the next generation. Over multiple
154 generations the target autosomes will be introgressed onto the SR X chromosomes (Supplementary
155 figure 3).

156 **Dissection of male testes**

157 We examined the size of male testes and seminal vesicles (SV's) of SR males, ^{Supp}SR males and ST
158 males. To look for changes to male testes that may develop over time as they mature or
159 alternatively be present when just sexually mature and disappear with age, we dissected males
160 when they were either just sexually mature (at 3-days-old) or when they had reached full sexual
161 maturity (at 7-days-old) (48). When at either 3- or 7-days-old, males were anesthetised using FlyNap
162 (Carolina Biological Supply Co., Burlington, USA) and the testes were dissected out of the males in
163 300µl of phosphate buffered saline (PBS). Once dissected, the testes were transferred onto a
164 microscope slide containing 80µl of PBS. A photograph of the testes was taken under constant light
165 conditions with a microscope mounted Nikon D5100 camera at x40 magnification. To control for

166 body size effects, the left wing of each male was removed using forceps under a microscope
167 (alternatively, the right wing was taken if the left was damaged) and mounted onto a glass
168 microscope slide. A photograph of each wing was taken using the microscope mounted camera.

169 **Measuring abnormalities to testes**

170 We first examined photographs for evidence of abnormalities in the testes. Only abnormalities
171 in the testes themselves was considered. Abnormality was scored as a binary variable by the
172 presence of physical holes in the orange sheath that coats the testes (Figure 1a). An intact orange
173 sheath that showed thinning was not classed as abnormal. To avoid bias and ensure consistency, this
174 process was double blinded and each photo was independently evaluated by three people. Here, the
175 proportion of males that showed abnormal testes differed by observer. However, when repeating
176 the analysis just using the data of each observer, we found the same effect that ^{Supp}SR males
177 experienced more abnormalities (Analysis 1: GLM: $F_{8,210} = 11.227$, $p < 0.001$; Analysis 2: GLM: $F_{8,210} =$
178 9.821, $p < 0.001$; Analysis 3: GLM: $F_{8,210} = 8.818$, $p < 0.001$) (Supplementary Figure 4). Thus, a
179 composite of the three analyses was used to determine a final testis abnormality score for each male
180 in which testes were assigned as abnormal if two or more evaluators scored them as abnormal.

181 **Measuring testes size, SV size, accessory gland size and wing length**

182 Testes and SV size was measured from images using ImageJ 1.48 (49). A subset of images with
183 visible accessory glands were also analysed in ImageJ to measure accessory gland size. The area of
184 each individual testis was measured (Figure 1b) and a mean of the two taken for each male. If only
185 one testis could be measured for size, it was this single measurement that represented a male's
186 mean testes size. The same procedure was performed for the SV's and accessory glands. Wing
187 lengths were also measured in ImageJ to control for body size (Figure 1b), using the method in
188 Gilchrist *et al.,* 2001 (50). The mean testes, SV and accessory gland size for each male was divided by
189 wing length to generate testes, SV or accessory gland size relative to wing length. These relative
190 measurements were used in all statistical analyses.

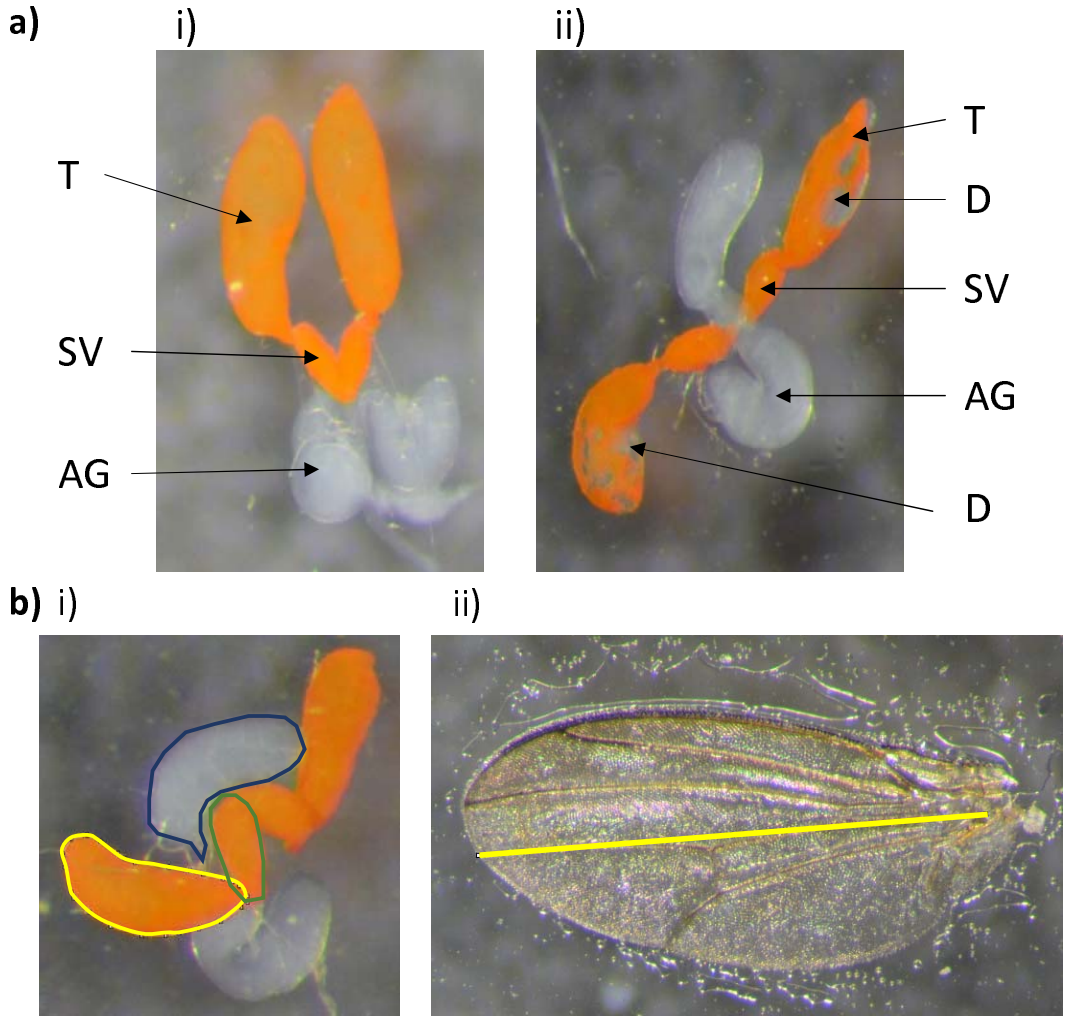


Figure 1: Analysis and measurement of male reproductive organs and wing size. a) Example images of testes (T), seminal vesicles (SV), accessory glands (AG) and visible abnormalities to testes (D). ai) Image of intact testes with no abnormalities to the orange sheath. aii) Abnormal testes defined by the presence of physical holes in the orange sheath. bi) Measurement of testes (yellow), seminal vesicles (green) and accessory gland (blue) area using ImageJ. bii) Measurement of wing length in ImageJ as defined in Gilchrist *et al.,* 2001 (50).

Statistical analysis

All statistical analysis was performed using the statistical software R, version 3.6.0 (51). A binomial generalised linear model (GLM) was used to investigate differences in the frequency of abnormalities to male testes. Testes size, SV size and accessory gland size were analysed by fitting a GLM with a gaussian error distribution. All models considered the effects of the type of male (SR, ^{Supp}SR, ST susceptible or ST suppressed males), and the age of the male when it was dissected (3- or

223 7- days old). ST males were split into either ST susceptible or ST suppressed to allow comparisons of
224 the suppressor in the absence of the selfish X chromosome. For testes abnormalities, testes size, and
225 SV size analysis, the original location of origin of the SR X chromosome was included in the model as
226 this provided a better model fit. In these analyses, the effect of SR X chromosome location was
227 significant but idiosyncratic and does not affect the main effects of drive (for individual line effects,
228 see supplementary figures 7-10). For all GLMs, a fully saturated model was produced and was
229 systematically reduced to the minimum adequate model through a step-wise simplification process
230 using Akaike information criterion (AIC). Difference in responses to all GLMs under different
231 treatments were assessed by analysis of variance (ANOVA) followed by a TukeyHSD comparison. For
232 simplicity, all graphs presented align SR next to ST susceptible and ^{Supp}SR next to ST suppressed as
233 they share genetic backgrounds to make interpretation clearer of what happens when SR is added to
234 each genetic background.

235 ## Results

236 **Wing size**

237 Wing size (a proxy for body size), was analysed using a GLM. The model considered the type
238 of male and the age of the male and their interaction. The interaction was not significant and was
239 dropped from the model. Wing size was affected by age (GLM: $F_{1, 218} = 7.518$, $p = 0.007$), where 7-
240 day-old males were smaller than 3-day-old males (TukeyHSD, $p = 0.007$). The type of male also had a
241 significant effect on wing size (Supplementary figure 6: GLM: $F_{3, 218} = 32.762$, $p < 0.001$). ST
242 suppressed males were smaller than ^{Supp}SR (TukeyHSD, $p = 0.002$), SR (TukeyHSD, $p < 0.001$) and ST
243 susceptible males (TukeyHSD, $p < 0.001$). ^{Supp}SR males were smaller than SR and ST susceptible males
244 (TukeyHSD, $p < 0.001$ and $p = 0.002$, respectively). There was no difference in the wing size of SR and
245 ST susceptible males (TukeyHSD, $p = 0.120$).

246 **Testes size**

247 Mean testes size relative to wing length was analysed using a GLM. The model considered
248 the type of male and the age of the male. A significant age by type of male interaction was found
249 (Figure 2a: GLM: $F_{3, 213} = 3.663$, $p = 0.0132$). Here, ST males (both suppressed and susceptible) have
250 significantly reduced testes size as they age from 3-days-old to 7-days-old (TukeyHSD, $p < 0.001$ for
251 both ST types). This reduction in testes size was not seen in SR and ^{Supp}SR males as they aged
252 (TukeyHSD, $p = 0.975$, TukeyHSD, $p = 0.780$, respectively). There was an overall effect of age on
253 testes size (GLM: $F_{1, 213} = 15.705$, $p < 0.001$), where 7-day old-males had larger testes (TukeyHSD, p
254 <0.001).

255 **Seminal vesicle size**

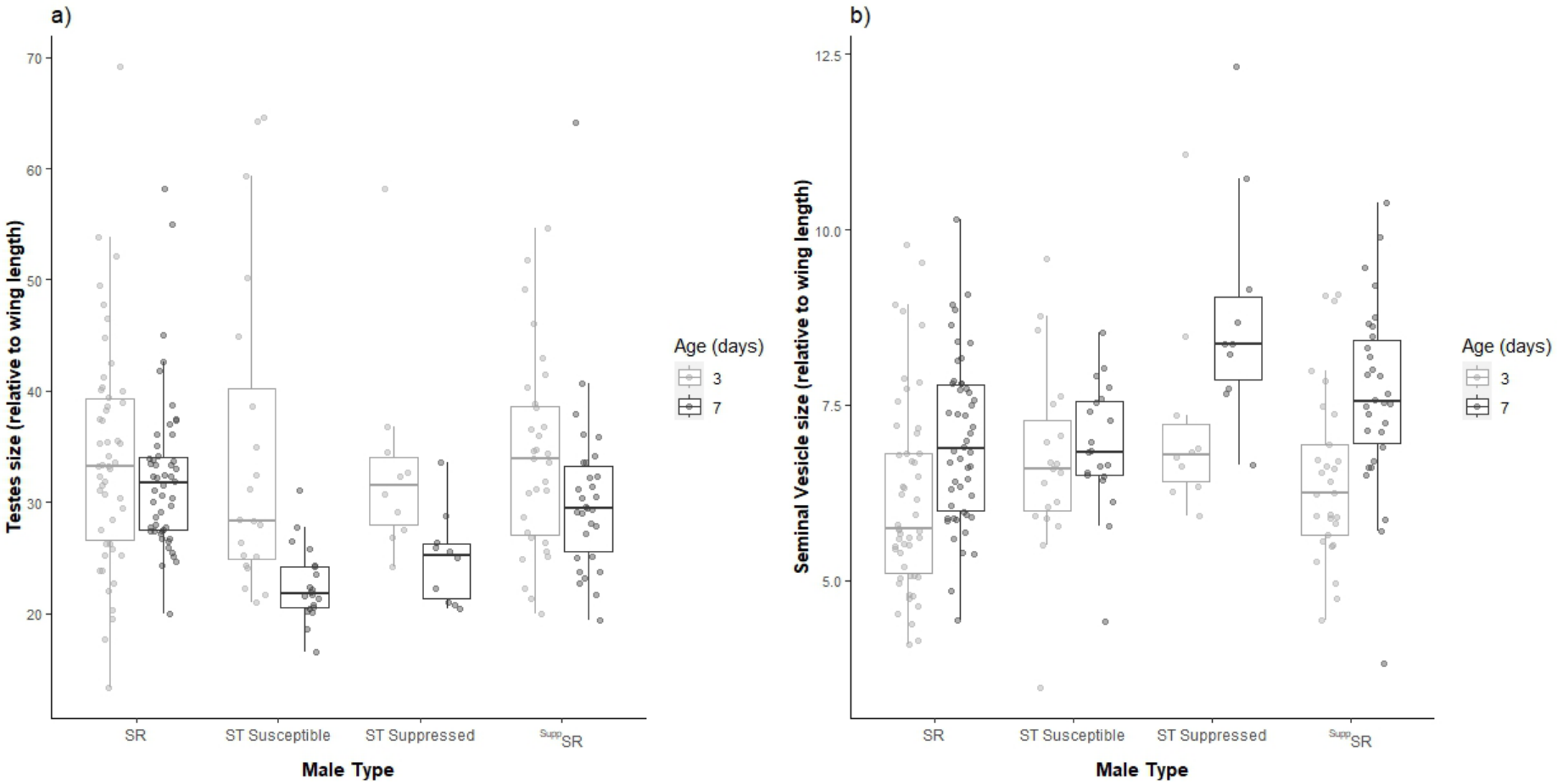
256 Mean SV size relative to wing length was analysed using a GLM. The model considered the
257 type of male and the age of the male and their interaction. The interaction was not significant, so
258 was dropped from the model. Overall, SV's were bigger in 7-day-old males than 3-day-old males
259 (Figure 2b: GLM: $F_{1, 213} = 24.095$, $p < 0.001$). The type of the male also had a significant effect on

260 mean SV size (Figure 3b: GLM: $F_{3, 213} = 7.384$, $p < 0.001$). Here, ST suppressed males have larger SV's

261 than SR males (TukeyHSD, $p < 0.01$), ST susceptible males (TukeyHSD, $p = 0.005$) and ^{Supp}SR males

262 (TukeyHSD, $p = 0.026$). However, there was no difference in SV size between ^{Supp}SR and SR and ST

263 susceptible males.



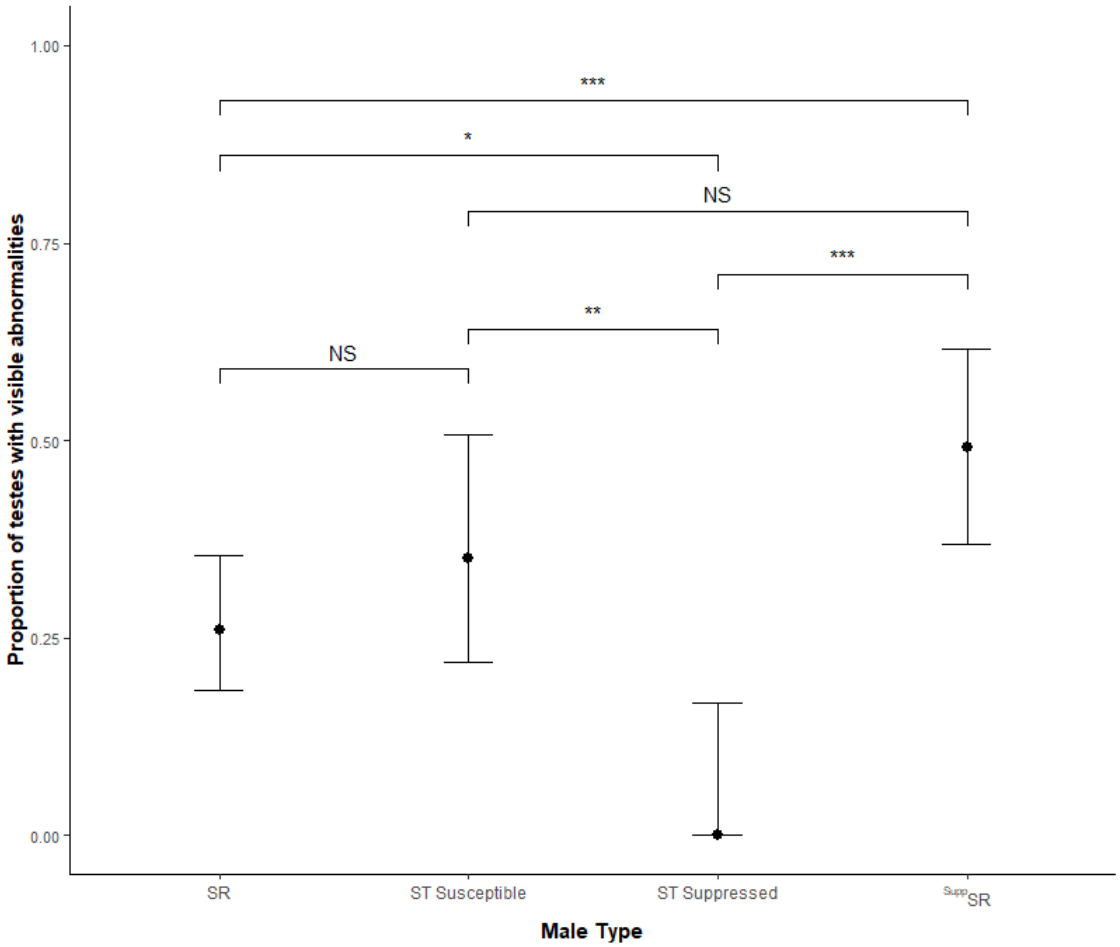
264

265 **Figure 2:** The mean size of a male's two testes (a) or seminal vesicles (b) of *Drosophila subobscura* males that were either SR, ST susceptible, ST suppressed
266 or ^{Supp}SR. All measurements were relative to individual body size. The boxplots display the upper and lower quartiles, the median and the range. Individual
267 points represent each measurement obtained.

268 **Abnormal testes**

269 The presence of visible abnormalities to testes was analysed using a binomial GLM. The
270 model considered the type of male and the age of the male and their interaction. Neither age nor
271 the age by male type interaction had an effect on visible abnormalities to a male's testes (GLM: $F_{1, 199}$
272 $= 0.064$, $p = 0.801$, $F_{10,199} = 1.609$, $p = 0.106$, respectively) so they were dropped from the model.
273 Male type had a significant effect on the likelihood of male having abnormal testes (Figure 3: GLM:
274 $F_{3, 210} = 9.580$, $p < 0.001$). While most categories differed from one another, the most noticeable
275 differences were that ^{Supp}SR males had a significantly higher proportion of abnormal testes
276 compared to ST suppressed males (where by our measure, 49% of ^{Supp}SR males had visibly abnormal
277 testes in comparison to 1% ST suppressed males) (Figure 3: TukeyHSD, $p < 0.001$). On the other
278 hand, SR males did not differ significantly in the frequency of abnormal testes in comparison to ST
279 susceptible (18% of SR males had damage to their testes in comparison to 22% in ST susceptible
280 males, TukeyHSD, $p = 0.594$). ^{Supp}SR also had a higher amount of damage than SR males (TukeyHSD, p
281 $= 0.0015$). An example of both normal and abnormal testes can be seen in Figure 1a.

282 We also dissected the testes from SR, ^{Supp}SR and ST susceptible males when 7-days-old for
283 preliminary and exploratory analysis of the internal structure of each male type's testes (see
284 supplementary figure 11 for details). SR and ^{Supp}SR testes showed disorganisation, with gaps
285 between cells, while ST susceptible males showed normal tightly packed sperm development
286 (supplementary figure 11). While ^{Supp}SR males showed a higher level of organisation than SR males,
287 the internal structure of male testes did not resemble that of ST susceptible males.



288 **Figure 3:** The mean proportion of males that have visible abnormalities to their testes that were
289 either SR males, ST susceptible males, ST suppressed males or ^{Supp}SR males, with 95% confidence
290 intervals. The figure also reports statistical comparisons where NS is non-significant, * is $p < 0.05$, **
291 is $p < 0.01$ and *** is $p < 0.001$.

292 **Accessory gland size**

293 Accessory glands were measured in any images where they were clearly visible. Mean
294 accessory gland size relative to wing length was analysed using a GLM. The model considered the
295 type of male and the age of the male. Models analysing accessory gland size did not consider the
296 individual location the SR X chromosome originated from due to the smaller sample size and having
297 little power to detect differences. A significant age by type of male interaction was found (Figure 4:
298 GLM: $F_{3, 143} = 3.689$, $p = 0.0135$). Here, ^{Supp}SR males' accessory glands increase in size as they age
299 (TukeyHSD, $p = 0.004$). This change in accessory gland size was not seen in SR males (TukeyHSD, $p =$
300 0.999), ST susceptible males (TukeyHSD, $p = 0.999$) or ST suppressed males (TukeyHSD, $p = 0.933$).
301 Male type also had an effect on accessory gland size (GLM: $F_{3, 143} = 3.689$, $p = 0.0135$). Overall, ^{Supp}SR
302 males had larger accessory glands than SR and ST susceptible and ST suppressed males (TukeyHSD, p
303 $= 0.046$, TukeyHSD, $p < 0.001$, TukeyHSD, $p = 0.037$, respectively). SR males also had larger accessory
304 glands than ST susceptible males (TukeyHSD, $p = 0.003$). Finally, the age of the male had an effect on
305 accessory gland size (GLM: $F_{1, 143} = 5.713$, $p = 0.0181$). Overall, 7-day-old males had larger accessory
306 glands than 3-day-old males (TukeyHSD, $p = 0.019$).

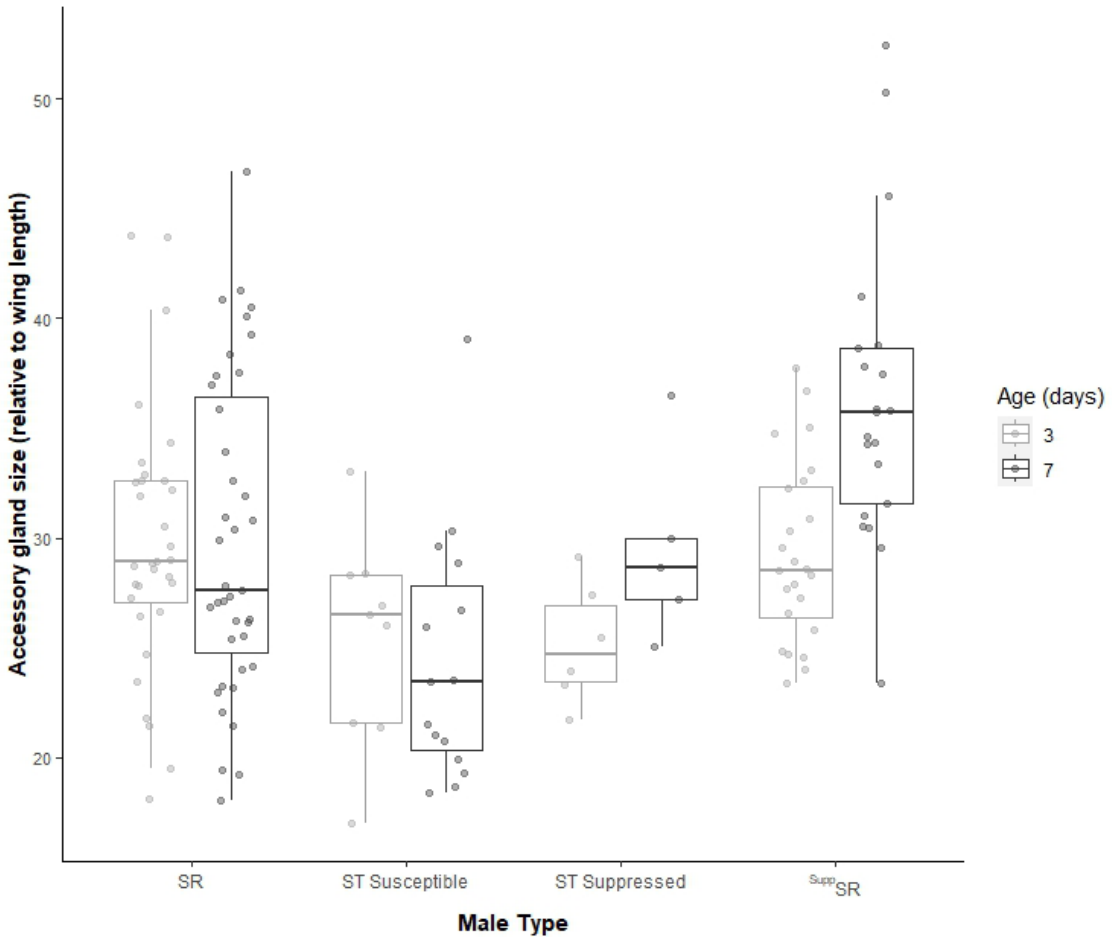


Figure 4: The mean size of the two accessory glands of male *Drosophila subobscura* that were either SR, ST susceptible, ST suppressed or ^{Supp}SR. All measurements were relative to individual body size. The boxplots display the upper and lower quartiles, the median and the range. Individual points represent each measurement obtained.

311 ## Discussion

312 XCMD and their suppressors generate potent genetic conflict that may also be a force
313 influencing gonad development, morphology and evolution (31,32). In this study, we examined
314 whether the intragenomic conflicts created by SR and its suppressor affects gonad morphology in *D.*
315 *subobscura.* We found significant differences in testes, seminal vesicle, and accessory gland size
316 depending on whether a male carried XCMD or not, and also if XCMD was suppressed. We also
317 found the first evidence of extreme whole-organ abnormal development of testes associated with
318 carrying a suppressor of XCMD. While males with SR from independent origins had differences in the
319 morphological traits observed, this variation was idiosyncratic and overall the main effect of SR and
320 its suppressor were the same, suggesting these effects are robust across populations. In conclusion,
321 we found strong evidence that XCMD and its suppressor can have a major impact on gonad
322 development and morphology of its carriers.

323 ***Testes and Seminal Vesicle size***

324 We found evidence that the testes of ST (both suppressed and susceptible) males, but not
325 those of SR and ^{Supp}SR males, reduce in size as they age. *Drosophila subobscura* are strictly
326 monandrous (28,44), and males may not benefit from high investment in continually producing large
327 quantities of sperm (52–54). In particular, if *D. subobscura* can partition their ejaculates, like in
328 numerous species across a wide array of taxa (55,56), they may only transfer to a female the sperm
329 she will need. This results in reduced pressure on the testes to continually produce sperm (and the
330 costs of spermatogenesis this entails). In contrast to the reduction in testes size observed in ST
331 males, it appears that SR and ^{Supp}SR testes remain the same size as males age. We propose three
332 mutually non-exclusive reasons for this: (1) compensation, (2) damage or (3) a delay in sperm
333 production.

334 In stalk-eyed flies, XCMD males have enlarged testes to compensate drive induced sperm
335 mortality, and males produce an equivalent number of sperm to normal males (31). This

336 compensation of male testes appears to occur from an early development stage (32). Compensation
337 may also be the effect that we have observed in *D. subobscura*. Indeed, a previous study has
338 suggested that the loss of Y-bearing sperm in SR males is compensated through cyst doubling in the
339 testes (57,58). Unlike in stalk-eyed flies, we do not observe this increase in testes size at all ages. At
340 3-days-old, all male types have equivalent sized testes. If this was compensation, would SR male
341 carriers not always have enlarged testes? In addition, in a species with no sperm competition
342 (28,44), loss of sperm number may not need to be compensated.

343 Contrary to the compensation hypothesis, damage may instead be inflicted upon the testes
344 resulting in their enlarged size. The internal testes structure of SR and ^{Supp}SR males showed
345 disorganisation, with gaps between cells, while ST testes showed normal tightly packed sperm
346 development (supplementary figure 11). This may indicate that XCMD carriers instead experience
347 internal testes damage and/or spermatogenic failure, the severity of which may accumulate over
348 time. Indeed, the gapping between cells and their general disorganisation may explain why SR and
349 ^{Supp}SR male have enlarged testes at this age. However, a full investigation of the internal morphology
350 of testes and associated sperm development is required to explore this further. Currently, little is
351 known about the interaction of XCMD and genes generally involved in gonad development (59,60).

352 Finally, if testes are largest simply when the process of spermatogenesis is at its peak, then
353 sperm production, and the reaching of a full sperm complement, may simply be delayed in SR and
354 ^{Supp}SR males. As a result, testes would remain larger for a longer period of time, while they continue
355 full sperm production to an older age. A detailed temporal breakdown of sperm production and
356 storage would be required to confirm this hypothesis.

357 We found no strong effects of carrying XCMD on SV size, as these were equivalent in all male
358 types, other than ST suppressed. The reasons for this are unclear and do not provide strong support
359 for any of our above theories. SVs are sperm storage organs in *Drosophila* males, where mature
360 sperm that develop in the testes move into the SV for storage until mating (61,62). Thus, in unmated

361 males, SV size is generally related to the amount of sperm stored (63). The equivalent SV size may
362 indicate that these male types are all producing and storing a similar amount of sperm, providing
363 some additional support for the compensation theory. Conversely, all the sperm stored in the SVs of
364 SR and ^{Supp}SR males may not be viable. It is possible that these males are bundling dead or damaged
365 sperm that is incapable of fertilisation or zygote formation. The disruption to the internal structure
366 of these testes suggests that spermatogenesis is likely to be affected in these males (supplementary
367 figure 11). A full examination of the viability of sperm stored within the SVs would be required to
368 confirm this.

369 ***Whole testes abnormalities***

370 Remarkably, we found evidence that, when the suppressor is paired with a driving SR X
371 chromosome, it causes gross visible abnormalities to testes. Overall, these abnormalities are less in
372 XCMD males that lack the suppressor which is comparable to that of ST susceptible males. Once
373 evolved, suppressors against drive are usually expected to remove or minimise the negative impacts
374 of drive (33,64,65). However, we demonstrate for the first time that suppression, while mitigating
375 some negative effects of XCMD, can also have its own effects on testes morphology, that likely
376 imposes costs. In addition, given the scope of the morphological differences we see, suppression
377 genes may influence general testes development that, when expressed and paired with SR, results in
378 their abnormal development and potential spermatogenesis failures. In addition, these
379 abnormalities are not restricted to the external testes. Our preliminary SEM analysis of the internal
380 structure of male's testes shows that both SR and ^{Supp}SR males have a high level of disorganisation
381 and gapping between cells, while ST shows normal tightly packed sperm development. Thus, SR
382 appears to be having its own influence of testes morphology, that becomes enhanced when the
383 suppressor is also present. This is in support of our theory that SR and its suppressor are inflicting
384 damage or developmental changes upon a male's testes. To what effect, if any, these abnormalities
385 then has on a male's fertility remains an open question.

386 It is critical these abnormalities, and any associated costs is understood as it could affect the
387 balance between drivers and suppressor that may provide insight into the long-term stability of
388 drive that we see in many natural systems (43,66–68). Additionally, if suppressors can spread
389 despite deleterious effects, they have the potential to fix negative mutations within a population,
390 which is rarely considered in empirical studies. There are many cases of cryptic (or muted) drive
391 systems that are fixed within populations (64,65,69). Here, drive systems are only revealed through
392 outcrosses with other populations or species that do not have the suppressor. These factors are
393 especially important to consider in relation to artificial drive systems (70). What would happen to
394 artificial drive if a suppressor unexpectedly evolved and fixed deleterious mutations within the
395 target population? The evidence we have found that major abnormalities are associated with
396 suppression against XCMD suggests that not only can SGEs carry huge costs, but in addition
397 suppressors and their interactions with SGEs must be accounted for.

398 ***Accessory gland size***

399 The extent of changes to male gonads as a result of XCMD seem not to just be limited to the
400 testes. We observed changes to the whole of the male gonads, including the accessory glands.
401 Accessory glands are organs that are responsible for the production of seminal fluid that contains
402 accessory gland proteins (71). In *Drosophila*, accessory gland proteins influence female behaviour
403 and a males sperm competitive ability (72,73). In some systems, accessory gland size is also
404 correlated with male mating rate and success (74,75). In this study, we found that ^{Supp}SR accessory
405 glands are the largest, followed by SR which were larger than ST (both suppressed and susceptible)
406 males. Carrying SR may be associated with compensatory increases in investment in accessory gland
407 proteins to ensure their mating success rather than transferring an equivalent amount of sperm to
408 ST males. Additionally, the highest investment in accessory glands was from ^{Supp}SR. When SR males
409 are suppressed they also endured the highest frequency of testes abnormalities, which may indicate
410 males are investing more in gaining a mate. Despite this, it remains unclear as to what the role of

411 accessory glands is in a monogamous species where the importance of them becomes largely
412 unknown.

413 ***Conclusion***

414 Here, we demonstrate that the presence of XCMD and its suppressor can cause significant
415 morphological changes to male gonads. This is the second system, that we are aware of, where
416 XCMD strongly influences male gonad development (31,32). Unlike the XCMD system in stalk-eyed
417 flies, where morphological changes were deemed a compensation mechanism against drive, our
418 evidence suggests that XCMD in *D. subobscura* inflicts gross abnormalities on male gonads that
419 becomes even more severe in the presence of its suppressor. This highlights that SGEs are a strong
420 evolutionary force that can broadly impact gonad development and gametogenesis. Additionally,
421 considering drive alone may only reveal half of the story. Suppressors can also have their own severe
422 effects on their carriers. Overall, the fitness effects of either an SGE or its suppressors may be severe
423 for individuals that carry them.

424

425 Acknowledgements

426 The authors would like to thank Jola Tanianis for her help with the isolation of the SR chromosomes,
427 and Kieran Romain for measuring the accessory glands. The authors acknowledge the use of the
428 biomedical electron microscopy unit provided by Liverpool shared research facilities, Faculty of
429 Health and Life Sciences, University of Liverpool. We would also like to thank the three anonymous
430 referees for their helpful comments on the manuscript. This work was funded by the National
431 Environment Research Council ACCE DTP and the grant no. NE/S001050/1.

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