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

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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 affects 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 extreme whole-organ damage to testes that is specifically associated with a suppressor of XCMD. Unlike other studies, our evidence suggests that XCMD in *D. subobscura* inflicts extreme damage on male gonads. This damage is most severe 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 testes 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 organisms normal chromosome complement, that carry superfluous DNA (10,11). They exhibit super non-Mendelian inheritance, allowing them to selfishly accumulate in the germline (10). As a result, they are widely reported in nature and can cause phenotypic differences and affect 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 the germline (14). Strong evidence for the role of SGEs in evolution comes from observation of the consequences of SGE presence in 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 males 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 gonads, respond to deal with these challenges?

58 Here we investigate how the development of the testes respond when faced with XCMD and
59 a suppressor of this system. XCMD is a type of SGE that creates huge conflicts within the testes (21).
60 XCMD manipulates the process of meiosis in males, where selfish X chromosomes kill Y-bearing
61 sperm during spermatogenesis, resulting in the biased production of daughters (17). The
62 transmission advantage gained by XCMD can allow it to rapidly spread through populations,
63 distorting sex ratios and even leading to population extinction owing to a lack of males (22–24).
64 Thus, testes in XCMD carrying males are a good model to investigate how SGEs impact the evolution
65 of gonads. Additionally, testes are particularly vulnerable to SGEs due to the asymmetry of gamete
66 production. Eggs are large and very costly for a female to produce, so anything that damages eggs
67 will be extremely costly and very quickly selected against. On the other hand, because male gametes
68 are generally massively over-produced, damage to sperm may be more readily be tolerated. In cases
69 where males only produce a reduced amount of sperm, they can still be successful, making sperm
70 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, XCMD in stalk eyed flies even appears to have
74 resulted in the response to the loss of sperm created by drive through compensation (30–32). Here,
75 males carrying XCMD have an increased testes size that allows them to produce an equivalent
76 amount of sperm to standard males. These studies, that look at how the testes respond to drive,
77 raises the question as to whether drive 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 paired with a
81 driving X chromosome (34). Once a suppressor has evolved, it is expected to rapidly spread to
82 fixation (35,36). Despite this, the impact of suppressors of XCMD on the testes themselves has been
83 rarely considered in empirical studies, despite being recognised as potentially important in
84 theoretical studies (37). Ongoing co-evolution between drive and its suppressor acting within the
85 testes could have different outcomes depending on how a suppressor works, whether it has
86 associated costs, and if it has non-target effects. Thus, the question remains does the emergence of
87 suppressors negate any testes phenotype (and selection) produced by SGEs. Alternatively, the need
88 for testes to evolve could be enhanced if they have to deal with both XCMD and suppression, which
89 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_{sub}' (41). The strength of drive
92 is strong with SR_{sub} carrying males producing broods that are 85-100% female (41,42). Given this
93 strong drive, a simple expectation is that SR_{sub} should rapidly spread to fixation everywhere.
94 However, it does not. Instead, in North Africa, SR_{sub} has remained at a stable frequency of
95 approximately 20% for at least 50 years (41–43). Additionally, *D. subobscura* are reported to be
96 strictly monandrous (28,44) so effects of sperm competition are irrelevant in this species (18,45). We
97 recently discovered suppressors of the SR_{sub} system (43) that are capable of fully restoring a normal
98 50:50 sex ratio to SR_{sub} carrying males. Despite the prediction that suppressors against drive should
99 rapidly fix within populations, this suppressor remains rare at a frequency of 1-5% (46). The lack of
100 spread of this suppressor may indicate that it is associated with its own costs and creates its own
101 burden upon its host.

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

109 ## Materials and Methods

110 **Origin and maintenance of fly stocks**

111 Isofemale lines (or “isolines") are a method for capturing genetic variation from a wild
112 population by inbreeding the offspring of single wild caught females to produce a genetically
113 invariant line (47). Isolines of *Drosophila subobscura* were originally collected from Tabarka, Tunisia
114 (36.95°N 8.74°E) in 2013 (48), Ras el Oud, Morocco (34.15°N 4.00°W), Demnat, Morocco (31.73°N
115 7.01°W) and Ouzoud, Morocco (32.02°N 6.72°W). Moroccan collections took place in 2019 and all
116 flies were caught using banana, yeast and beer baited traps (49). All isolines were kept in standard
117 *Drosophila* vials on an ASG double yeast medium (10g agar, 85g sucrose, 40g yeast extract, 60g
118 maize, 1000ml H_2O, 25ml 10% nipagin) at 18°C on a 12:12 light/dark cycle. Experimental flies were
119 generated by collecting unmated flies from isolines from each population. All eclosed flies were
120 removed from populations 24 hours prior to ensure flies had not mated. Fly collection was done
121 using CO_2 anaesthesia to separate flies by sex. Flies were stored in new vials on a sugar yeast
122 medium (100g sugar, 100g yeast extract, 20g agar, 1000ml H_2O, 25ml 10% nipagin, 5ml propionic
123 acid) with no more than 10 flies per vial.

124 **Isolation and introgression of SR_{sub} X chromosomes**

125 We isolated an SR_{sub} chromosome from a wild caught fly from each of Ras el Oud, Morocco,
126 Demnat, Morocco and Ouzoud, Morocco, where SR_{sub} occurs at a frequency of approximately 20%
127 (43). SR_{sub} males were initially identified though a phenotypic scan by counting their offspring sex
128 ratio. Males that produced more than 85% females were deemed SR_{sub} males and were used to
129 isolate their driving X chromosomes. In order to produce the SR_{sub} males (that carry an SR_{sub}
130 chromosome), suppressed SR_{sub} males ($^{Supp}SR_{sub}$), that carry a suppressed version of the SR_{sub}
131 chromosome, and standard (ST) males (that do not carry the SR_{sub} chromosome) that were used in
132 this investigation, we introgressed both vulnerable and suppressing ST isolines onto the isolated
133 SR_{sub} chromosomes. This introgression process meant we were able to produce flies that only differ

134 genetically in carrying drive (males either carried an SR_{sub} X chromosome or an ST X chromosome)
135 and either carried the suppression genes or genes susceptible to drive. Preliminary experiments
136 have shown that the Moroccan isolines show no suppression against SR_{sub} (43), thus these isolines
137 were used to produce the SR_{sub} males that produce 100% daughters. Additionally, the ST isoline from
138 Tunisia contains genes that are capable of suppressing SR_{sub}. This Tunisian ST isoline was therefore
139 used to produce the $^{Supp}SR_{sub}$ males that produce a normal 50:50 sex ratio despite the male carrying
140 SR_{sub}.

141 We introgressed the SR_{sub} X chromosomes onto either the vulnerable Moroccan ST isolines or
142 the suppressing Tunisian ST isoline for 14 to 17 generations. The introgression process was done in
143 two stages. First, females homozygous for the SR_{sub} X chromosome ('SRSR' females) were crossed to
144 a standard ('ST') male from the target isoline. This cross produces heterozygous females ('SRST'
145 females) that are discarded, and 'SR_{sub}' males that carry the SR_{sub} X chromosome and the target
146 isoline autosomes. These SR_{sub} males are then crossed to SRSR females to produce the next
147 generation of introgressed SRSR females (Supplementary figure 1).

148 **Dissection of male testes**

149 We examined the size of male testes and seminal vesicles (SV's) of SR_{sub} males, $^{Supp}SR_{sub}$ males
150 and ST males. To look for changes to male testes that may develop over time as they mature or
151 alternatively be present when just sexually mature and disappear with age, we dissected males
152 when they were either just sexually mature (at 3 days old) or when they had reached full sexual
153 maturity (at 7 days old) (50). When at either 3- or 7-days-old, males were anesthetised using FlyNap
154 (Carolina Biological Supply Co., Burlington, USA) and the testes were dissected out of the males in
155 300µl of phosphate buffered saline (PBS). Once dissected, the testes were transferred onto a
156 microscope slide containing 80µl of PBS. A photograph of the testes was taken under constant light
157 conditions with a microscope mounted Nikon D5100 camera at x40 magnification. In total, between
158 30 and 50 males were dissected for each treatment (see supplementary Table 1). To control for body
159 size effects, the left wing of each male was removed using forceps under a microscope (alternatively,

160 the right wing was taken if the left was damaged) and mounted onto a glass microscope slide. A
161 photograph of each wing was taken using the microscope mounted camera.

162 **Measuring damage to testes**

163 We first examined photographs for evidence of visible damage to the testes. Only damage to the
164 testes themselves was considered. Damage was scored as a binary variable by the presence of
165 physical holes in the orange sheath that coats the testes (Figure 1a). An intact orange sheath that
166 showed thinning was not classed as damage. To avoid bias and ensure consistency, this process was
167 double blinded and each photo was independently evaluated by three people. Here, the proportion
168 of males that experienced damage to their testes differed by observer. However, when repeating
169 the analysis just using the data of each observer, we found the same effect that $^{Supp}SR_{sub}$ males
170 experienced more damage than both SR_{sub} and ST males (Analysis 1: GLM: $F_{8,210} = 11.227$, $p < 0.001$;
171 Analysis 2: GLM: $F_{8,210} = 9.821$, $p < 0.001$; Analysis 3: GLM: $F_{8,210} = 8.818$, $p < 0.001$) (Supplementary
172 Figure 2). Thus, a composite of the three analyses was used to determine a final testes damage score
173 for each male in which testes were assigned as damaged if two or more evaluators scored them as
174 damaged.

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

176 Testes and SV size was measured from images using ImageJ 1.48 (51). A subset of images with
177 visible accessory glands were also analysed in ImageJ to measure accessory gland size. The area of
178 each individual testis was measured (Figure 1b) and a mean of the two taken for each male. If only
179 one testis could be measured for size, it was this single measurement that represented a male's
180 mean testes size. The same procedure was performed for the SV's and accessory glands. Wing
181 lengths were also measured in ImageJ to control for body size (Figure 1b), using the method in
182 Gilchrist *et al.,* 2001 (52). The mean testes, SV and accessory gland size for each male was divided by
183 wing length to generate testes, SV or accessory gland size relative to wing length. These relative
184 measurements were used in all statistical analyses.

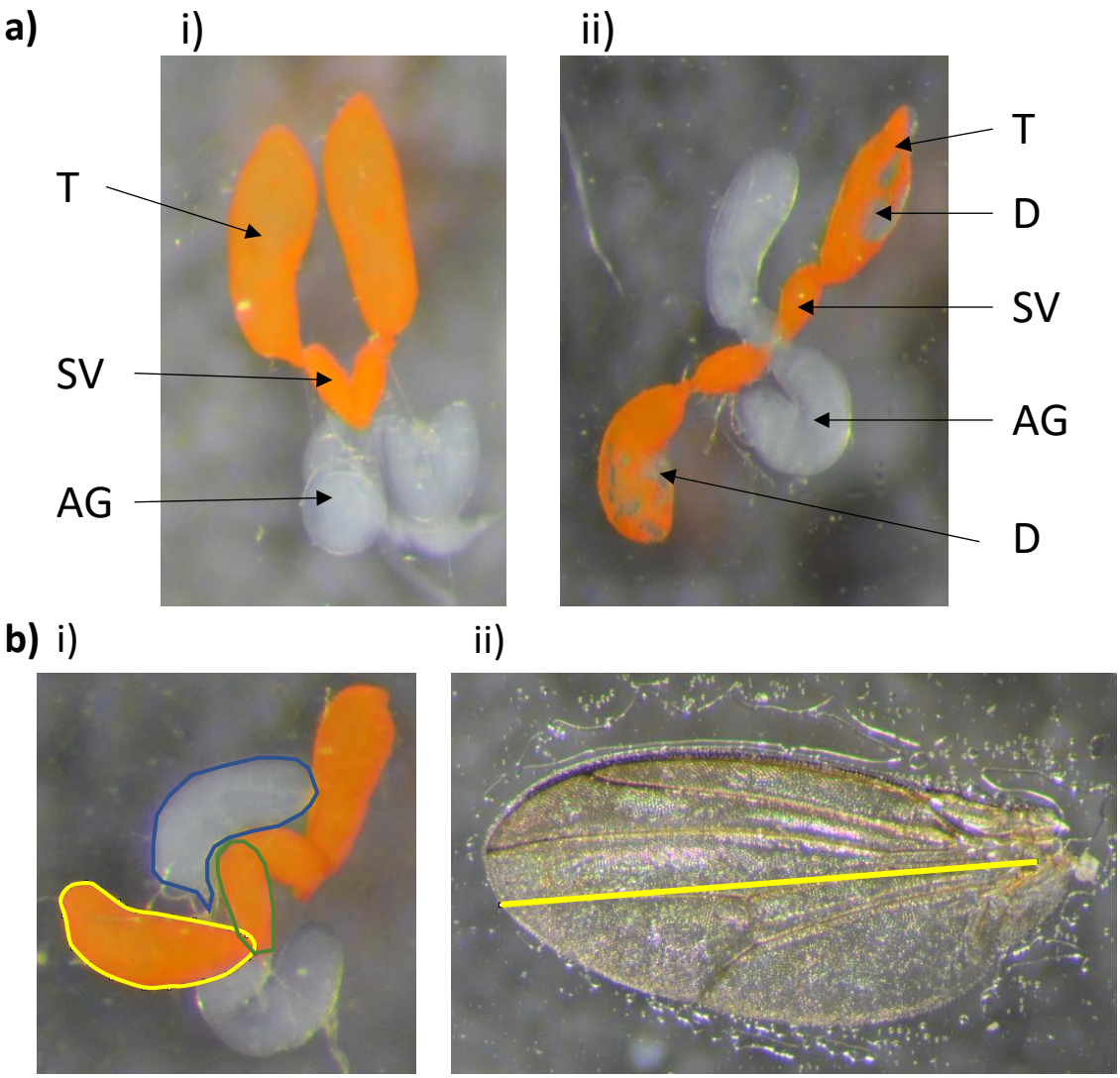

Figure 1: Analysis and measurement of male reproductive organs and wing size. a) Analysing testes images for evidence of damage where testes (T), seminal vesicles (SV), accessory glands (AG) and damage to testes (D) are all visible. ai) Image of intact testes with no damage to the orange sheath. aii) Damaged 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 (52).

Statistical analysis

All statistical analysis was performed using the statistical software R, version 3.6.0 (R Core Team, 2019). A binomial generalised linear model (GLM) was used to investigate differences in damage 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_{sub}, $^{Supp}SR_{sub}$ or ST males), and the age of the male when it was dissected (3- or 7 days old). For the damage, testes

size and SV size analysis, the original location of origin of the SR_{sub} X chromosome was included in the model as this provided a better model fit. In these analyses, the effect of SR_{sub} X chromosome location was significant but idiosyncratic and does not affect the main effects of drive (for individual line effects, see supplementary figures 5-8). For all GLMs, a fully saturated model was produced and was systematically reduced to the minimum adequate model through a step-wise simplification process using Akaike information criterion (AIC). Difference in responses to all GLMs under different treatments were assessed by analysis of variance (ANOVA) followed by a TukeyHSD comparison.

224 ## Results

225 **Wing size**

226 Wing size (a proxy for body size), was analysed using a GLM. The model considered the type
227 of male and the age of the male and their interaction. The interaction was not significant and was
228 dropped from the model. Wing size was affected by age (GLM: $F_{1,219} = 6.412$, $p = 0.012$). Whether a
229 male was SR_{sub}, $^{Supp}SR_{sub}$ or was ST also had a significant effect on wing size (Supplementary figure 4:
230 GLM: $F_{2, 219} = 25.471$, $p < 0.001$). SR_{sub} males were bigger than both $^{Supp}SR_{sub}$ males (TukeyHSD, $P <$
231 0.001) and ST males (TukeyHSD, $p < 0.001$). However, there was no difference in wing length
232 between ST and $^{Supp}SR_{sub}$ males (TukeyHSD, $p = 0.615$).

233 **Testes size**

234 Mean testes size relative to wing length was analysed using a GLM. The model considered
235 the type of male and the age of the male. A significant age by type of male interaction was found
236 (Figure 2a: GLM: $F_{2, 215} = 5.210$, $p = 0.00617$). Here, ST males have significantly reduced testes size as
237 they age from 3-days-old to 7-days-old (TukeyHSD, $p < 0.001$). This reduction in testes size was not
238 seen in SR_{sub} and $^{Supp}SR_{sub}$ males as they aged (TukeyHSD, $p = 0.919$, TukeyHSD, $p = 0.630$,
239 respectively).

240 **Seminal vesicle size**

241 Mean SV size relative to wing length was analysed using a GLM. The model considered the
242 type of male and the age of the male and their interaction. The interaction was not significant, so
243 was dropped from the model. Overall, SV's were bigger in 7-day-old males than 3-day-old males
244 (Figure 2b: GLM: $F_{1, 214} = 22.762$, $p < 0.001$). Whether a male carried SR_{sub}, $^{Supp}SR_{sub}$ or was ST also had
245 a significant effect on mean SV size (Figure 3b: GLM: $F_{2, 214} = 5.145$, $p = 0.00657$). Here, ST males have
246 larger SV's than SR_{sub} males (TukeyHSD, $p = 0.00950$). However, there was no difference in SV size
247 between $^{Supp}SR_{sub}$ and SR_{sub} males (TukeyHSD, $p = 0.066$) or between $^{Supp}SR_{sub}$ and ST males
248 (TukeyHSD, $p = 0.797$).

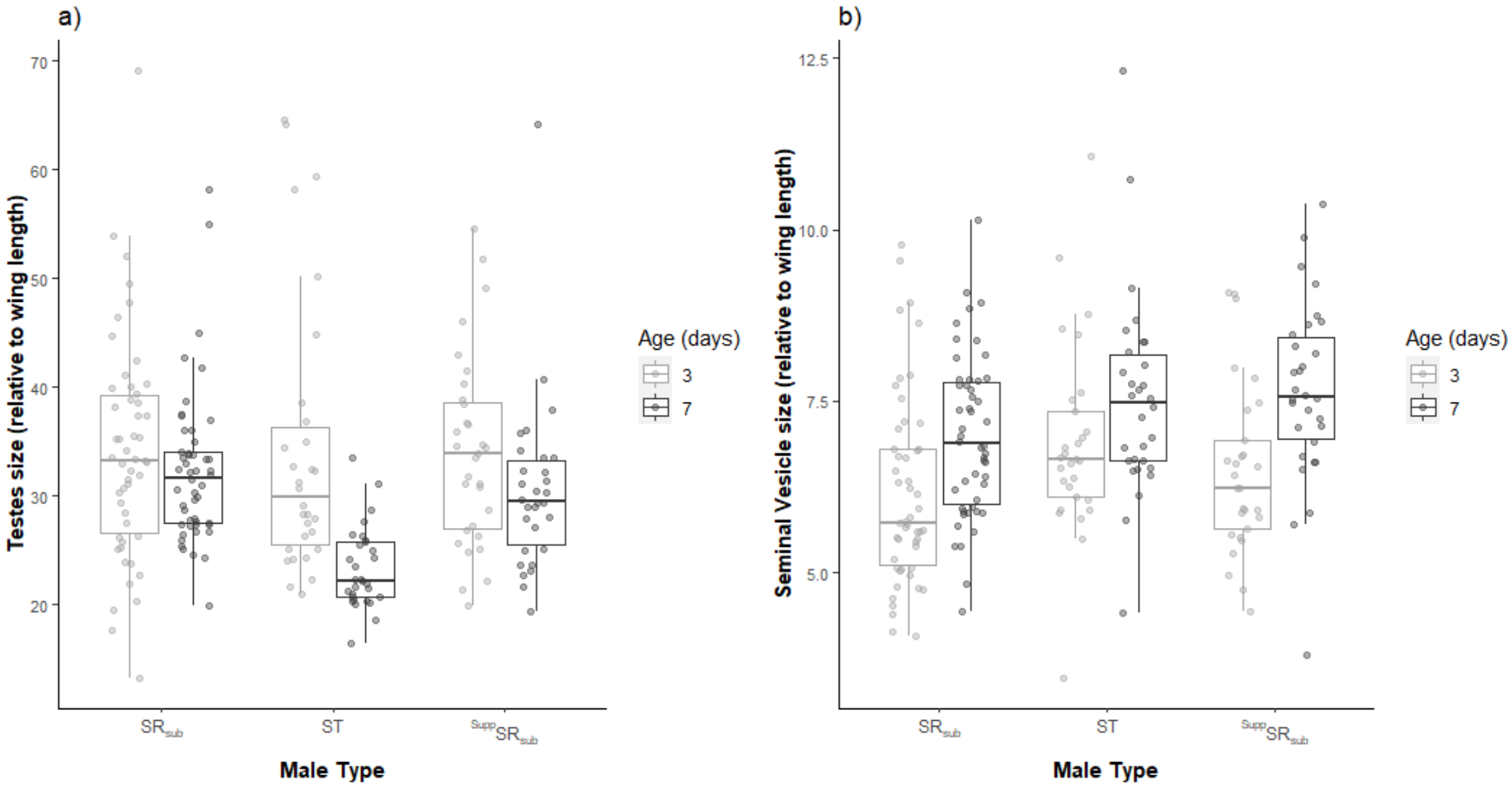

Figure 2: The average size of a male's two testes (a) or seminal vesicles (b) of *Drosophila subobscura* males that were either SR_{sub}, ST or $^{Supp}SR_{sub}$ relative to body size. The boxplots display the upper and lower quartiles, the median and the range. Individual points represent each measurement obtained.

252 **Damage to testes**

253 Differences in damage to testes was analysed using a binomial GLM. The model considered
254 the type of male (ST; SR_{sub}; $^{Supp}SR_{sub}$) and the age of the male (3 or 7 days old) and their interaction.
255 Neither age nor the interaction age by male type interaction had an effect on the amount of damage
256 to a male's testes (GLM: $F_{1, 209} = 0.0669$, $p = 0.791$) so they were dropped from the model. Whether
257 the male was SR_{sub}, $^{Supp}SR_{sub}$ or ST had a significant effect on the amount of damage that occurred to
258 the testes (Figure 3: GLM: $F_{2, 210} = 8.837$, $p < 0.001$). $^{Supp}SR_{sub}$ males had a significantly higher
259 proportion of damaged testes than SR_{sub} and ST males (where by our measure, 49% of $^{Supp}SR_{sub}$ males
260 had visibly damaged testes in comparison to 23% of ST males and 26% of SR_{sub} males) (Figure 3:
261 TukeyHSD, $p < 0.001$, TukeyHSD, $p < 0.001$, respectively). There was no difference in the amount of
262 damage to testes between ST and SR_{sub} males (Figure 3: TukeyHSD, $p = 0.905$). An example of both
263 damaged and undamaged testes can be seen in Figure 1a.

264

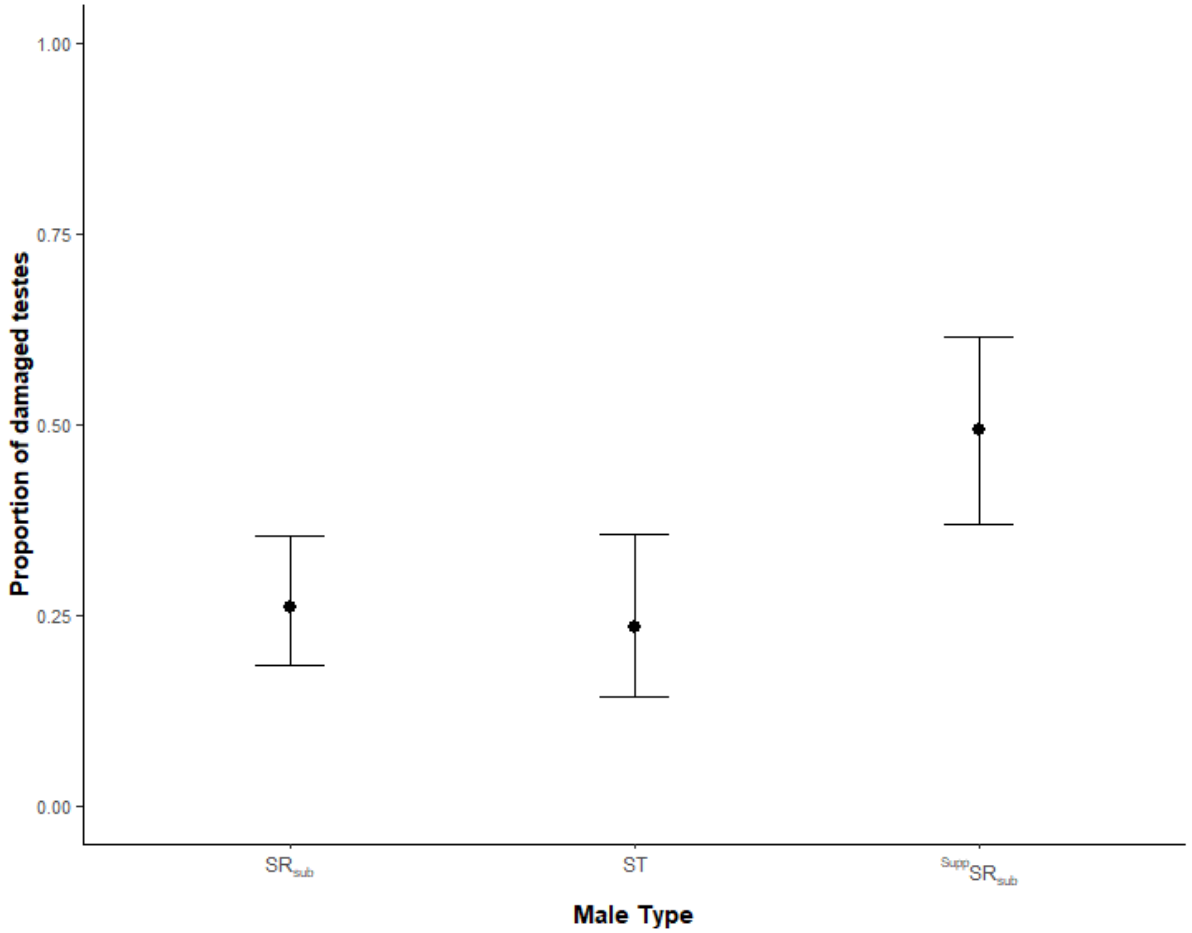

Figure 3: The mean proportion of males that have visible damage to their testes that were either SR_{sub} males, ST males or $^{Supp}SR_{sub}$ males, with 95% confidence intervals.

Accessory gland size

Accessory glands were measured in any images where they were clearly viable. Mean accessory gland size relative to wing length was analysed using a GLM. The model considered the type of male and the age of the male. Models analysing accessory gland size did not consider the individual location the SR_{sub} X chromosome originated from due to the smaller sample size and having little power to detect differences. A significant age by type of male interaction was found (Figure 4: GLM: $F_{2, 145} = 4.950$, $p = 0.00833$). Here, $^{Supp}SR_{sub}$ males' accessory glands increase in size as they age (TukeyHSD, $p = 0.00207$). This change in accessory gland size was not seen in SR_{sub} males (TukeyHSD, $p = 0.999$) or ST males (TukeyHSD, $p = 0.999$). Whether the male carried SR_{sub}, $^{Supp}SR_{sub}$ or was ST also had an effect on accessory gland size (GLM: $F_{2, 145} = 13.570$, $p < 0.001$). Overall, $^{Supp}SR_{sub}$ males had larger accessory glands than both SR_{sub} and ST males (TukeyHSD, $p = 0.045$, TukeyHSD, $p < 0.001$, respectively). SR_{sub} males also had larger accessory glands than ST males (TukeyHSD, $p = 0.0021$). Finally, the age of the male had an effect on accessory gland size (GLM: $F_{1, 145} = 5282$, $p = 0.022$). Overall, 7-day-old males had larger accessory glands than 3-day-old males (TukeyHSD, $p = 0.023$).

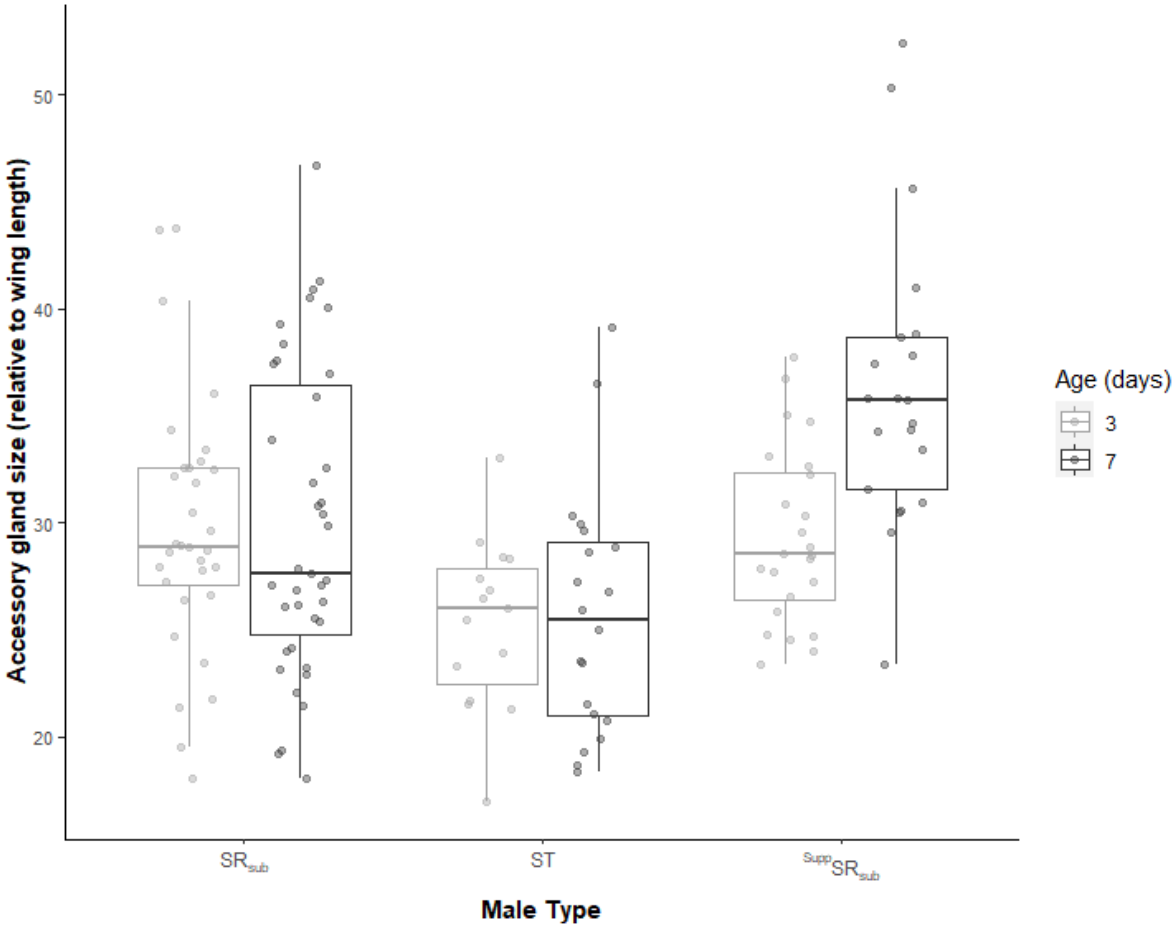

Figure 4: The mean size of accessory glands of male *Drosophila subobscura* that were either SR_{sub}, ST or $^{Supp}SR_{sub}$ relative to body size. A significant age by interaction term was found where $^{Supp}SR_{sub}$ males accessory glands increase in size as they age. This effect was not seen in SR_{sub} or ST males.

288 ## Discussion

289 XCMD and their suppressors generate potent genetic conflict that may also be a force
290 influencing gonad development, morphology and evolution (31,32). In this study, we examined
291 whether the intragenomic conflicts created by SR_{sub} and its suppressor affects gonad morphology in
292 *D. subobscura.* We found significant differences in testes, seminal vesicle, and accessory gland size
293 depending on whether a male carried XCMD or not, and also if XCMD was suppressed. We also
294 found the first evidence of extreme whole-organ damage to testes associated with carrying a
295 suppressor of XCMD. While males with SR_{sub} from independent origins had differences in the
296 morphological traits observed, this variation was idiosyncratic and overall the main effect of SR_{sub}
297 and its suppressor were the same, suggesting these effects are robust across populations. In
298 conclusion, we found strong evidence that XCMD and its suppressor can have a major impact on
299 gonad development and morphology of its carriers.

300 ***Testes and Seminal Vesicle size***

301 We found evidence that the testes of ST males, but not those of SR_{sub} and $^{Supp}SR_{sub}$ males,
302 reduce in size as they age. *Drosophila subobscura* are strictly monandrous (28,44), and males may
303 not benefit from high investment in continually producing large quantities of sperm (53–55). In
304 particular, if *D. subobscura* can partition their ejaculates, like in numerous species across a wide
305 array of taxa (56,57), they may only transfer to a female the sperm she will need, rather than their
306 whole ejaculate. This results in reduced pressure on the testes to continually produce sperm (and
307 the costs of spermatogenesis this entails). SVs are sperm storage organs in *Drosophila* males, where
308 mature sperm that develop in the testes move into the SV for storage until mating (58,59). Thus, in
309 unmated males, SV size is generally related to the amount of sperm stored (60). The combination of
310 ST males' testes reducing in size and having larger SVs suggest that these males initially produce
311 more sperm that fill their SVs, before subsequent investment in sperm production is reduced. In
312 contrast to the reduction in testes size observed in ST males, it appears that SR_{sub} and $^{Supp}SR_{sub}$ testes

313 remain the same size as males age. We propose three mutually non-exclusive reasons for this: (1)
314 compensation, (2) damage or (3) a delay in sperm production.

315 In stalk-eyed flies, males that carry XCMD have enlarged testes to compensate drive induced
316 sperm mortality, and XCMD males produce an equivalent number of sperm to normal males (31).
317 The compensation of male testes also appears to occur from an early development stage (32).
318 Compensation may also be the effect that we have observed in *D. subobscura*. Indeed, a previous
319 study has suggested that the loss of Y-bearing sperm in SR_{sub} males is compensated through cyst
320 doubling in the testes (61,62). Additionally, SR_{sub} males are capable of producing an equivalent
321 number of offspring to ST males in single matings, although this could be attributed to other factors,
322 such as increased mating duration or females not being sperm limited in this context (28). However,
323 the smaller SVs observed in SR_{sub} suggests that these males may actually be producing and storing
324 fewer sperm, which is not consistent with the compensation of sperm production.

325 Although bigger than SR_{sub} males, SVs of $^{Supp}SR_{sub}$ were still smaller than ST males. A key
326 feature of suppression is that it must prevent some of the killing of Y sperm (33). As $^{Supp}SR_{sub}$ males
327 have at least some of their Y sperm protected, this result suggests they produce more sperm than
328 SR_{sub}. However, the suppressor may not fully rescue all Y sperm or it may be associated with its own
329 damaging effects on sperm production. The smaller SVs of both types of XCMD carrying males in
330 comparison to ST males indicates fewer sperm are made and stored by these males, again
331 challenging the idea of compensation in this system. In addition, as there is no sperm competition in
332 *D. subobscura* (28,44), loss of sperm number may not need to be compensated.

333 The above discussion presumes testes size reflects functional capacity. Alternately, damage
334 to testes may be causing cellular damage, and this is preventing the reduction in testes size as males
335 age. Any damage that is occurring within the testes, either through the killing of Y-bearing sperm or
336 by carrying the suppressor, could result in inflammation or the accumulation of stress proteins at the
337 site of damage as males age. Necrotic cell death may additionally contribute to the testes continued

338 enlargement via a loss of plasma membrane integrity followed by cytoplasmic leakage (63,64). This
339 could lead to the cells swelling, producing inflamed tissues as a consequence. This is in contrast to
340 programmed apoptotic cell death that is characterised by cell shrinkage and membrane blebbing
341 (64,65). Thus, the lack of reduction in size may be more likely to be a combination of factors
342 associated with damage that is occurring in the testes.

343 Delays in sperm production could also affect the differences in testes sizes and SV sizes we
344 observe in our male types. If testes are largest simply when the process of spermatogenesis is at its
345 peak, then sperm production, and the reaching of a full sperm complement, may simply be delayed
346 in SR_{sub} and $^{Supp}SR_{sub}$ males. As a result, testes would remain larger for a longer period of time, while
347 they continue full sperm-production to an older age. Currently, little is known about the interaction
348 of XCMD and genes generally involved in gonad development (66,67). SR_{sub} males having the
349 smallest SVs, followed by $^{Supp}SR_{sub}$ males, and ST males having the largest SVs would also be
350 consistent with this development delay, with XCMD males that are suppressed potentially showing
351 an intermediate timing to reach full ejaculate complement. A closer examination of the fitness of
352 males of different ages, under repeated mating treatments, might reveal if developmental delays in
353 sperm production could explain the observed difference in testes morphology and whether these
354 delays influence male fitness.

355 ***Whole testes damage***

356 Remarkably, we found evidence that, when the suppressor is paired with a driving X SR_{sub}
357 chromosome, it causes extreme physical damage to testes. Overall, this damage is less in XCMD
358 males that lack the suppressor and comparable to standard males. Once evolved, suppressors
359 against drive are usually expected to remove or minimise the negative impacts of drive (33,68,69).
360 However, we demonstrate for the first time that suppression, while mitigating some negative effects
361 of XCMD, can generate its own damage (and presumably, costs). In addition, given the scope of the
362 morphological differences we see, suppression genes may influence general testes development

363 that, when expressed and paired with SR_{sub}, results in their damage or developmental failures. To
364 what effect, if any, this damage then has on a male's fertility remains an open question.

365 XCMD is known and expected to impose fitness costs upon its carriers (18,45,70). Despite
366 this, less is known about how suppressors evolve to respond and if suppression is costly (33). Thus,
367 there is currently an imbalance in our knowledge, where we have a deeper understanding of the
368 costs of drive, but less so of the suppressors themselves and the balance between drive and
369 suppression. If costs of the suppressor are strong enough, suppressors may not spread. Hence, the
370 suppressor remains rare in populations where SR_{sub} is present (46). In spite of this, the suppressor
371 has not been lost from populations. This could be because the suppressor genes only appear costly
372 when paired with XCMD, and our data is consistent with this. Thus, partial suppression may allow
373 XCMD to persist for long periods of time (41,43). To fully understand the extent of this damage to
374 gonads, we need to; (1) determine if this damage is altering the internal structure of the male
375 gonads; (2) see the extent of cell death within XCMD carriers that are both suppressed and
376 unsuppressed; and (3) use an omics approach to investigate difference in the proteomes (71).

377 It is critical this damage is understood as it could affect the balance between drivers and
378 suppressor that may provide insight into the long-term stability of drive that we see in many natural
379 systems (43,72–74). Additionally, if suppressors spread despite deleterious effects, they have the
380 potential to fix negative mutations within a population, which is rarely considered in empirical
381 studies. There are many cases of cryptic (or muted) drive systems that are fixed within populations
382 (68,69,75). Here, drive systems are only revealed through outcrosses with other populations or
383 species that do not have the suppressor, suggesting that suppression has become fixed. These
384 factors are especially important to consider in relation to artificial drive systems (76). What would
385 happen to artificial drive if a suppressor unexpectedly evolved and fixed deleterious mutations
386 within the target population? The evidence we have found that major damage is associated with

387 suppression against XCMD suggests that not only can SGEs carry huge costs, but in addition
388 suppressors and their interactions with SGEs must be accounted for.

389 ***Accessory gland size***

390 The extent of changes to male gonads as a result of XCMD seem not to just be limited to the
391 testes. We observed changes to the whole of the male gonads, including the accessory glands.
392 Accessory glands are organs that are responsible for the production of seminal fluid that contains
393 accessory gland proteins (77). In *Drosophila*, accessory gland proteins influence female behaviour
394 and a males sperm competitive ability (78,79). In some systems, accessory gland size is also
395 correlated with male mating rate and success (80,81). In this study, we found that $^{Supp}SR_{sub}$ accessory
396 glands are the largest, followed by SR_{sub} which were larger than ST males. Carrying SR_{sub} may be
397 associated with compensatory increases in investment in accessory gland proteins to ensure their
398 mating success rather than transferring an equivalent amount of sperm to ST males. Additionally,
399 the highest investment in accessory glands was from $^{Supp}SR_{sub}$. When SR_{sub} males are suppressed they
400 also endured the highest amount of damage to their testes, which may indicate males are ensuring
401 their mating success as they endure fitness costs in other ways. Despite this, it remains unclear as to
402 what the role of accessory glands is in a monogamous species where the importance of them
403 becomes largely unknown.

404 ***Conclusion***

405 Here, we demonstrate that the presence of XCMD and its suppressor can cause significant
406 morphological changes to male gonads. This is the second system, that we are aware of, where
407 XCMD strongly influences male gonad development (31,32). Unlike the XCMD system in stalk-eyed
408 flies, where morphological changes were deemed a compensation mechanism against drive, our
409 evidence suggests that XCMD in *D. subobscura* inflicts extreme damage on male gonads that
410 becomes even more severe in the presence of its suppressor. This highlights that SGEs are a strong
411 evolutionary force that can broadly impact gonad development and gametogenesis. Additionally,

412 considering drive alone may only reveal half of the story. Suppressors can also have their own severe
413 effects on their carriers. Overall, the fitness effects of either an SGE or its suppressors may be severe
414 for individuals that carry them.

415

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