A test of genetic models for the evolutionary maintenance of same-sex sexual behaviour

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Summary

The evolutionary maintenance of same-sex sexual behaviour (SSB) has received increasing attention because it is perceived to be an evolutionary paradox. The genetic basis of SSB is almost wholly unknown in non-human animals, though this is key to understanding its persistence. Recent theoretical work has yielded broadly-applicable predictions centred on two genetic models for SSB: overdominance and sexual antagonism. Using *Drosophila melanogaster*, we assayed natural genetic variation for male SSB and empirically tested predictions about the mode of inheritance and fitness consequences of alleles influencing its expression. We screened 50 inbred lines derived from a wild population for male-male courtship and copulation behaviour, and examined crosses between the lines for evidence of overdominance and antagonistic fecundity selection. Consistent variation among lines revealed heritable genetic variation for SSB, but the nature of the genetic variation was complex. Phenotypic and fitness variation was consistent with expectations under overdominance, although predictions of the sexual antagonism model were also supported. We found an unexpected and strong paternal effect on the expression of SSB, suggesting possible Y-linkage of the trait. Our results inform evolutionary genetic mechanisms that might maintain low but persistently-observed levels of male SSB in *D. melanogaster*, but highlight a need for broader taxonomic representation in studies of its evolutionary causes.

Keywords: *Drosophila melanogaster*, evolutionary genetics, overdominance, quantitative genetics, same-sex sexual behaviour, sexual antagonism

1. Introduction

Studies of same-sex sexual behaviour (SSB) have focused on a diverse range of animal taxa, from deep sea squid to insects [1-6]. The core of such research hinges on the assumption that SSB imposes a direct fitness cost on individuals that express it, and therefore represents an "evolutionary paradox" demanding explanation (e.g. [7-10]). However, SSB is no different from any other trait that might appear inexplicably costly when benefits are not immediately obvious. Historically, similar traits have included aggression, altruism and sexual ornamentation [11].

Characterising the genetic basis of SSB in a broad range of species is critical to better understanding its evolutionary persistence, but biologists studying non-human animals are hampered by a lack of empirical genetic data. We are aware of only a pair of artificial selection experiments using the flour beetle *Tribolium castaneum* [12-13], a report of intersexual correlation for SSB in the seed beetle *Callosobruchus maculatus* [14], plus a number of candidate gene studies in *Drosophila melanogaster* that document male-male courtship as an incidental effect of mutations affecting sex recognition (see [3] or [6] for reviews). The latter have elegantly illuminated proximate neurogenetic mechanisms that influence the expression of SSB in *Drosophila*, but they have limited power to explain the evolutionary forces that shape this complex, quantitative trait in natural populations [15]. The deficit of genetic data on SSB in non-human animals is compounded by the limited number of theoretical studies that quantitatively model its genetic basis (reviewed in [4], see also [16-20]).

Recent theoretical work by Gavrilets and Rice [16] formulated explicit predictions to detect modes of selection maintaining SSB. Their models focus on two genetic hypotheses

for SSB – overdominance and sexual antagonism – that have garnered recent attention in the literature, though their conceptual origins date at least to the 1950s [4,16,21]. The Gavrilets and Rice [16] models are formulated in the context of human sexual orientation, but they are applicable to SSB in any diploid dioecious organism. Under overdominance, costly SSB could be maintained in a population if alleles that increase an individual's tendency to exhibit SSB in the homozygous state confer a balancing fitness advantage when expressed in heterozygotes. In contrast, sexual antagonism could maintain costly SSB if alleles increasing its expression in one sex cause a countervailing fitness advantage when expressed in the opposite sex. The hypotheses are not mutually exclusive, and they yield predictions about the inheritance and fitness effects of alleles influencing SSB (Table 1).

Here we empirically test predictions outlined by Gavrilets and Rice [16]. We used the *Drosophila* Genetic Reference Panel (DGRP) [22], which consists of inbred *Drosophila melanogaster* lines originally derived from the wild. Male-male courtship in *D. melanogaster* is well-documented, it occurs in wild-type flies at low but persistent levels, and SSB phenotyping protocols have been developed and validated [23,24]. SSB in insects is often thought to be caused by poor sex recognition [6,25,26]. In *D. melanogaster*, flies express sex-specific cuticular hydrocarbons (CHCs) and wild-type flies can detect and differentiate these cues [27]. We designed our study to minimise misidentification that can occur when young adult flies have not yet developed sex-specific CHC profiles, because we were interested in SSB that occurs despite the presence of cues for sexual identity [28].

First, we screened inbred lines to establish the existence of genetic variation for SSB. Second, we identified and validated lines showing consistently high levels of SSB ("high-SSB") and lines showing consistently low levels of SSB ("low-SSB") for use in crosses. Third, we performed experimental crosses using these high and low lines to test predictions about

84 parental contributions to offspring SSB and levels of dominance. Finally, we estimated
85 female fecundity, an important fitness component, from the crosses to test predictions
86 about the fitness of different genotypic combinations under each model. Our results reveal
87 inheritance patterns and fitness effects that provide mixed support for both models, but in
88 aggregate are most consistent with overdominance. We also uncovered an unexpected
89 paternal effect on the expression of SSB.
90

91 2. Materials and Methods

92 (a) Origin and maintenance of fly lines

93 We used 50 inbred lines from the *Drosophila* Genetic Reference Panel (DGRP) as focal test
94 flies in same-sex sexual behaviour (SSB) assays. The DGRP was derived from a wild
95 population in Raleigh, North Carolina, USA. Lines were subjected to a minimum of 20
96 generations of full-sib mating and have an estimated inbreeding coefficient of F = 0.986 [22],
97 although this is now likely an underestimate owing to their maintenance in laboratory
98 culture for additional generations after 2012. It is likely that rare allelic variants were lost
99 during the production of the inbred lines, limiting the power to detect small effect loci in
100 association studies [29]. However, this means that any phenotypic differences we found in
101 our screen represent a conservative assessment of genetic variation for male SSB.
102 Establishing which lines show consistent variation in male SSB enabled us to then perform
103 crosses and evaluate modes of inheritance and fitness effects.
104 We used an additional *D. melanogaster* strain carrying a yellow-body mutation on a
105 wild-type background, Hmr^2, as a consistent genotype against which to test DGRP
106 individuals in paired trials. The yellow-body strain was used so that each fly within a vial

could be distinguished and assigned specific behaviours. Hmr^2 flies originated from the Bloomington Stock Center (FlyBase ID: FBal0144848) [30]. The yellow-body mutation could conceivably exert pleiotropic effects on behavioral traits [31], although prior work suggests this is not likely to have a strong effect in our trials [24]. Furthermore, we avoided confounding our experimental design by always pairing focal DGRP flies with the Hmr^2 strain.

Stock flies were kept in large vials (25mm x 95mm) on cornmeal agar medium seeded with yeast. They were maintained at 18 °C on a 12:12 light:dark photoperiod. During experiments, virgin males were collected under light CO_2 anaesthesia from stock vials, whereupon they were transferred individually to small vials (16mm x 95mm) and allowed to recover. Experimental flies were kept at 23 °C until they were used in assays. We were specifically interested in situations where the sex of interacting partners was unambiguous and readily detectable, so we only used virgin yellow-body males 3-5 days old and virgin DGRP males 6-8 days old in SSB trials.

(b) Initial SSB screen and validation

Some of the data below have been reported in a previous study focusing on indirect genetic effects on male tapping behaviour in yellow-body flies [32]. These data are the tapping behaviour of yellow males and orienting, following, tapping, licking, singing, abdomen curling, and general activity of DGRP males, for both the initial screen and validation (Dryad doi:10.5061/dryad.d4s1k). Here we focus on variation in same-sex courtship elements exhibited by DGRP males while interacting with yellow-body partners in paired trials. We focused on male SSB only. Female sexual behaviour in *D. melanogaster* is generally assessed in the context of mate rejection, and while females will partly determine the outcome of

any male mating attempt, quantifying female courtship is problematic owing to the lack of observable active courtship elements such as can be readily scored in males [33]. We used the behavioural assay described in Bailey et al. [24] to quantify male SSB. All DGRP lines were screened against the common strain to enable comparison among the inbred lines. We quantified three male courtship behaviours that characterise same-sex sexual interactions: licking, singing and abdomen curling (i.e. attempted mounting). We also scored orienting, following, and tapping behaviours, but restrict our focus here to licking, singing and abdomen curling. The latter are unambiguously expressed during the context of opposite-sex courtship and copulation interactions, whereas orienting, following and tapping are known to function in non-sexual contexts such as aggression [34,35]. Detailed descriptions and links to videos of exemplar behaviours can be found in Bailey et al. [24].

We recorded behaviours exhibited by both the focal DGRP male and his interacting yellow partner using an interval sampling technique [24,32]. One DGRP male and one Hmr^2 male were introduced into a small (16mm x 95mm) vial oriented horizontally, and behaviour was observed for three minutes spread over three evenly-spaced one-minute observation periods. Five trials were run simultaneously under fluorescent interior lighting and indirect sunlight between 19.4 °C and 24.9 °C during morning hours. We performed 39 or 40 trials for each DGRP line. Five trials were excluded from analysis after it was discovered they were performed at too low a temperature (17.1 - 17.2 °C); their exclusion did not qualitatively affect the results.

To validate our behavioural assay, we repeated the above procedure on a subset of 8 DGRP lines, with the observer blind to line identity. We selected three validation lines that exhibited high levels of SSB in the initial assay, and four that exhibited low levels of SSB. One intermediate line (RAL_897) was selected as it showed an unusual pattern of reaction norm

155 variation in a different experiment (unpublished data). The selection was made only with
156 respect to the behaviours involved in SSB: licking, singing and abdomen curling.
157 Maintenance, rearing, and behavioural observations (n = 40 per line) were performed as
158 before.

159 We quantified SSB in each trial using a binary assessment of whether licking, singing
160 or abdomen curling occurred. If any of those behaviours were exhibited by a male during
161 the 3-minute trial period, then he received an SSB score of 1. We calculated line mean trait
162 values as the proportion of trials in which the DGRP male exhibited SSB. There are
163 advantages and disadvantages to using this system of quantifying behaviour [24]. Estimating
164 the intensity of SSB within different lines, i.e. the number of bouts of SSB during trials, had
165 the potential to create a bias owing to the proportionally heavier weighting of data from
166 lines in which very few males exhibited the behaviour. Following analysis of the validation
167 data, overall SSB line means for the 8 re-tested lines were calculated by combining the
168 original and validation data.

169

170 (c) Behaviour diallel

171 Our validation study indicated that SSB among lines could be consistently classified as "high-
172 SSB" or "low-SSB". We selected two lines that exhibited high levels of SSB (RAL_149 and
173 RAL_75) and two lines that exhibited low levels of SSB (RAL_223 and RAL_38) to perform a
174 complete diallel cross. The identities of these lines remained blind to the observer
175 throughout the diallel experiments. Maintenance and rearing procedures were as described
176 above. The complete diallel included diagonal (intra-line) and off-diagonal (inter-line)
177 crosses, including reciprocals. Each of the 16 crosses was established by housing 10 virgin
178 males and 10 virgin females from the designated parental lines. Virgin male F_1 offspring

179 from these crosses were collected and maintained individually in small (16mm x 95mm) vials
180 as before. For each of the 16 crosses, we performed behavioural observations on F_1 males (n
181 = 39-50 F_1 males per cross) using the same protocol with yellow-body males as a standard
182 strain against which to quantify the expression of SSB.
183

184 ## (d) Fecundity diallel

185 We estimated a component of female fitness by measuring early fecundity of the F_1 diallel
186 offspring. We set up F_2 crosses using the diallel F_1 offpsring as parents. Virgin male and
187 female full sibs were mated, with ten replicate full-sib matings set up for each of the 16
188 cross types. One day old virgin parents were kept in small vials (16mm x 95mm) for two days
189 to enable mating and oviposition, whereupon they were transferred to a fresh vial for an
190 additional two days and then removed. Once eclosion commenced, adults were counted
191 and sexed daily until no new adults were observed to eclose. Total offspring numbers were
192 calculated by pooling the counts across all collections for each cross replicate. Two blocks
193 were run approximately two weeks apart to allow for uncontrolled environmental effects.
194 While our estimate of fitness only captured early-life fecundity, early-life fitness appears to
195 be genetically correlated with later-life fitness in *D. melanogaster* [36].
196

197 ## (e) Analysis

198 Statistical analyses focused on (i) the correspondence between original and validation SSB
199 screens, (ii) inter-line variation in SSB, (iii) comparison of SSB levels in F_1 offspring from
200 diallel crosses, and (iv) fecundity differences among the diallel crosses. Analyses were
201 performed in Minitab v.12.21 and SAS v.9.3.

(i) We tested whether the subset of validated DGRP lines showed the same relative levels of male SSB in a blind validation block as they did in our original screen using a binary logistic regression with a logit link function. There were only two factor levels in "block", preventing accurate covariance estimates if modelled as a random effect, so we modelled it as a fixed effect. The subsequent experiments required us to cross lines that displayed high levels of SSB with lines that displayed low SSB. Because we selected high- and low-SSB lines to validate, we tested whether the 3 high-SSB and 4 low-SSB lines yielded consistently high and low estimates of SSB across experimental blocks by including "SSB level" and the "block x SSB level" interaction as fixed effects. To account for variation arising from lines within "high-SSB" and "low-SSB", we nested "line" within "SSB level". Temperature was included as a covariate. As a secondary verification that our measurement of line means for SSB was consistent across blocks, we regressed line mean SSB from the validation block on line mean SSB from the original block for all 8 re-tested lines.

(ii) Finding no evidence for experimental block effects, we assessed variation in SSB across all 50 lines, combining the original and validation data for those 8 lines that had been re-tested. We used a mixed-model binary logistic regression in which "line" was modelled as a random effect and temperature was included as a covariate. A logit link was used and degrees of freedom were estimated using the Satterthwaite method. We also estimated broad-sense heritability by calculating $H^2 = V_g/V_p$. We obtained V_g using variance components from a standard analysis of variance (ANOVA) where $V_g = (MS_a\text{-}MS_w)/[(1/a\text{-}1)(\Sigma N_i\text{-}\Sigma(N_i^2)/\Sigma N_i)]$ and MS_a is mean squares among groups, MS_w is means squares within groups, a is the number of groups, and N_i is each group size. V_p is the overall phenotypic variance.

225 (iii) SSB expression was compared among male F_1 offspring of our diallel crosses
226 using a binary logistic regression and a logit link. The aim was to estimate the relative
227 contributions of maternal vs. paternal genotypes to the expression of SSB in offspring, so
228 the model included “maternal line”, “paternal line” and the “maternal x paternal”
229 interaction as fixed factors. Temperature was modelled as a covariate. Parental lines were
230 not modelled as random effects for the same reasons given previously, and also because
231 they had been selected for use in planned contrasts between “low-SSB” and “high-SSB” lines
232 [37].
233 (iv) Fecundity and offspring sex ratio (daughters/total offspring) of the diallel families
234 was assessed using general linear models (GLMs). Offspring sex ratio data was natural log
235 transformed prior to analysis. The key comparison for testing our predictions was among
236 offspring from the four types of inter-line crosses, but we first evaluated the difference
237 between inbred crosses (diagonal of the diallel) and all outbred crosses (off-diagonals). We
238 therefore modelled “inbreeding” as a fixed effect with two factor levels. Experimental block
239 and its interaction with “inbreeding” were modelled as fixed effects. Because the same lines
240 were used in multiple crosses, we included maternal and paternal line identity as fixed
241 effects to assess the impact of “inbreeding” above and beyond any line-specific effects.
242 Including the interaction between maternal and paternal line identity was hindered by the
243 fact that we lacked data from one cross in one of the experimental blocks due to failed
244 matings, so it was not included.
245 Inbreeding was a major source of variation in fecundity, so we proceeded to examine
246 fecundity of the inter-line crosses only. A *post hoc* GLM was performed on the same dataset
247 excluding information from the inbred crosses. We tested for variation in fecundity among
248 high-high, high-low, low-high, and low-low inter-line crosses, modelled as “cross type”, and

249 included the interaction between “cross type” and “block”. The same model structures were
250 applied to the natural log transformed offspring sex ratio data. In both analyses, we
251 excluded data from replicates for which 3 or fewer offspring collections could be made (n =
252 13).
253

254 # 3. Results

255 ## (a) Behavioural screen and validation

256 We performed 2,320 behavioural trials. The interaction between “SSB level” and “block” in
257 our validation analysis was a key indicator of how consistently we were able to quantify
258 variation in SSB across blocks (Figure 1). We found neither a significant “block” effect (binary
259 logistic regression: Wald $\chi^2_{[1]} = 1.03$, P = 0.310), nor a significant “block x SSB level”
260 interaction (binary logistic regression: Wald $\chi^2_{[1]} = 0.0043$, P = 0.948), which provided
261 confidence that our scoring technique reliably distinguished high-SSB and low-SSB lines
262 across independent experiments. Line effects nested within each SSB level were similarly
263 non-significant (binary logistic regression: Wald $\chi^2_{[5]} = 4.91$, P = 0.427). As expected, the “SSB
264 level” term in our model indicated that high-SSB and low-SSB lines differed significantly
265 (binary logistic regression: Wald $\chi^2_{[1]} = 9.95$, P = 0.0016). Temperature did not affect the
266 expression of SSB (binary logistic regression: Wald $\chi^2_{[1]} = 0.66$, P = 0.415). We confirmed the
267 overall consistency of SSB measurements in a follow-up regression comparing all eight line
268 means in the original versus validation blocks, which showed variation in SSB among lines to
269 be positively correlated across experiments (linear regression: adjusted $r^2 = 0.461$, $F_{1,6} =$
270 6.98, P = 0.038).

271 We detected considerable variation in the expression of male SSB across the 50

tested DGRP lines. The proportion of trials in which males displayed SSB ranged from 0.0% to 42.5% (Figure 2) (mixed-model binary logistic regression: n = 2315, Z = 3.81, P < 0.001). As before, temperature did not affect SSB expression (mixed-model binary logistic regression: $F_{1,2268}$ = 1.36, P = 0.244). Broad sense heritability calculated across the lines using a standard ANOVA was H^2 = 0.11, but this is probably an underestimate owing to inflated within-group variance relative to among-group variance, caused by the binomial scoring of SSB.

(b) Behaviour diallel

The genotype of fathers, but not mothers, exerted a considerable influence on offspring SSB in diallel crosses: F_1 males expressed SSB patterns more similar to their father's line than their mother's line (Figure 3). The paternal contribution to offspring SSB expression is evident from a significant "paternal line" effect (binary logistic regression: Wald $\chi^2_{[3]}$ = 17.03, P = 0.001), while in contrast, "maternal line" did not influence offspring SSB expression (binary logistic regression: Wald $\chi^2_{[3]}$ = 5.26, P = 0.154). Any interaction between maternal and paternal genotypes did not appear to be strong (binary logistic regression: Wald $\chi^2_{[9]}$ = 15.83, P = 0.070), and temperature had no effect (binary logistic regression: Wald $\chi^2_{[1]}$ = 0.13, P = 0.720).

(c) Fecundity diallel

Fecundity of F_1 females derived from diallel crosses showed a complex pattern of inheritance (Figure 4A). As expected, there was a clear difference between inbred (diagonal) and outbred (inter-line) crosses (Table 2A). However, fecundity of inter-line crosses was greater for crosses between lines showing high values of SSB, and F1 crosses

between high and low SSB lines. Females from crosses involving two low-SSB parents produced on average 25 fewer offspring than those derived from crosses involving either one high-SSB and one low-SSB parent or two high-SSB parents. This key fecundity difference was significant in our *post-hoc* comparison examining only inter-line crosses (Table 2B, Figure 4A). Overall, fecundity differed across the two experimental blocks, but the non-significant "cross type x block" interaction indicated that differences among cross types occurred in a consistent direction (Table 2A). Both maternal and paternal line identities also affected F_1 female fecundity, and mothers from high-SSB lines produced more offspring than those from low-SSB lines (Figure 4A, Table 2A). Although the overdominance model classically predicts that crosses should be most extreme, our results, that crosses between different high SSB lines and high and low SSB lines have higher early fecundity, are compatible with directional overdominance maintaining SSB in this population.

Offspring sex ratio of mated F_1 females was generally unaffected by diallel cross type, although a significant block interaction suggested that patterns of cross-specific variation were inconsistent (Figure 4B, Tables 3A-B). The original maternal lineage did not affect sex ratio, but the original paternal lineage did (Figure 4B, Table 3A). Despite this paternally-induced variation, there was no discernible pattern linking offspring sex ratio to the level of SSB expressed in the paternal line (Figure 4B).

4. Discussion

We found considerable, and repeatable, variation in male SSB when we screened 50 inbred *D. melanogaster* lines, which confirms a heritable genetic basis for the trait. Like any other trait that potentially reduces fitness, the evolutionary maintenance of SSB requires a

318 countervailing fitness benefit. Genetic models of SSB [16] have illustrated that such a
319 benefit need not accrue to the individual expressing SSB, but can occur as a result of a
320 fitness advantage specific to the alleles influencing the expression of SSB.

321 Phenotypic and fitness patterns from diallel crosses among lines with the highest
322 and lowest SSB trait values supported predictions under both genetic models. The diallel
323 results involved only a sample of extreme lines, and assaying a wider range of female
324 fitness components might yield different results. Taken in aggregate, however, our results
325 lend more support to an overdominant fitness advantage occurring when alleles influencing
326 SSB are present in a heterozygous state in crosses between lines, as opposed to an
327 antagonistic advantage that is only revealed when such alleles are expressed in females. The
328 two models are in fact not mutually exclusive and SSB may be maintained by a combination
329 of mechanisms, a possibility that is highlighted by the fact that we did not find exclusive
330 support for a single model of SSB in *D. melanogaster*. Instead, we found a complex mix of
331 inheritance patterns and fitness effects, plus an unusual pattern of paternal effects on the
332 expression of male SSB.

333 A sexually antagonistic mode of selection maintaining male SSB predicts that we are
334 more likely to find X-linkage of loci influencing SSB [16]. We did not find this, as there was
335 no detectable maternal effect on the expression of SSB in sons from diallel crosses. This
336 model also predicts that females from high-SSB lines should experience increased fecundity
337 and contribute to a female-biased sex ratio, the latter owing to greater accumulation of
338 male-deleterious mutations on X chromosomes carrying SSB-increasing alleles. The first of
339 these predictions received support, but the latter did not. Fecundity was influenced by both
340 maternal and paternal genotypes; it was higher in crosses where the mother had a high-SSB
341 genotype (Figure 4A), which is what the sexual antagonism model predicts (Table 1).

342 Offspring sex ratio was influenced by paternal, but not maternal, genotype, but not in a
343 pattern that related to whether fathers were from high-SSB or low-SSB lines.
344 We also detected evidence consistent with the heterozygote fitness advantage
345 predicted by the overdominance model. When lines that carried alleles for high levels of SSB
346 were crossed, the resulting offspring had higher fecundity than those from low-low crosses.
347 In offspring from these crosses, heterozygosity at loci affecting SSB is expected because the
348 parents were derived from different high-SSB lines. Moreover, these effects were not driven
349 purely by heterosis, as we set up high-high and low-low crosses with different high-SSB and
350 low-SSB lines, respectively. Fecundity of offspring from low-low crosses was more than 15%
351 lower than the other crosses. However, sex-specific patterns of dominance in our data
352 might also be consistent with a model in which loci under sexually antagonistic selection are
353 X-linked [38]. Gavrilets and Rice [16] noted that under such a scenario, SSB is expected to be
354 recessive in the sex for which it reduces fitness, and dominant in the sex in which it
355 increases fitness. Consistent with this, male SSB appeared to show recessivity in our crosses
356 (Figure 3), whereas loci causing high male SSB had a dominant effect on female fitness in the
357 fecundity assay derived from those crosses (Figure 4a).
358 The paternal effects uncovered in our analyses of male SSB and offspring sex ratio
359 were unexpected and suggest a promising area for future research. Our screen of all 50
360 inbred lines revealed modest but significant broad-sense heritability. Crosses between a
361 subset of extreme lines confirmed this genetic variation for SSB by revealing a clear parent-
362 of-origin effect on offspring SSB levels , but surprisingly the paternal genotype exerted a
363 strong influence on the expression of male SSB and on offspring sex ratio while the maternal
364 genotype did not. Relatively few loci have been identified on the heterochromatic Y
365 chromosome of *D. melanogaster*, but those that have been studied appear to be strongly

366 implicated in male fitness [39]. In an analysis of polymorphic Y chromosomes crossed into a
367 common wild-type *D. melanogaster* background, Chippendale and Rice [40] found
368 substantial epistatic fitness effects of variation on the Y. Intriguingly, such male fitness
369 effects may arise from variation in sperm competition and mating behaviour. Several genes
370 with putative spermatogenesis functions have been characterised on the Y [41], and Y-
371 linked effects on male mating behaviours such as courtship song have been documented in
372 *D. virilis* [42]. Evidence for strong epistatic effects of Y-linked variation on patterns of
373 autosomal gene expression [43] suggests a mechanism whereby Y-linked variation
374 influences SSB: if balancing selection maintains polymorphism on the Y because of fitness
375 benefits in some genetic backgrounds but not others, detrimental epistatic fitness effects
376 mediated by the Y chromosome could manifest as high levels of male SSB. For example,
377 epigenetic modifications disrupting sexually dimorphic gene expression have been
378 suggested as a plausible mechanism underlying the development of SSB [17,44]. If genetic
379 variation on the Y is associated with male SSB, it might be productive to test which
380 autosomal genes interact with such Y-linked variation, and whether they are susceptible to
381 epigenetic modification.

382 Until further empirical work is performed, the diversity of genetic mechanisms
383 maintaining SSB will remain unknown. Such studies would benefit not only from focusing on
384 different systems, but also from expanding the scope of quantitative genetic experiments to
385 capture a broader range of genetic variation via inbred lines or pedigree-based animal
386 model approaches. Our study focused on male SSB because active courtship behaviour in *D.*
387 *melanogaster* is sex-limited, although it would be useful to perform similar genetic analyses
388 in species amenable to studying female SSB. Such work could clarify whether male and
389 female SSB are maintained by similar selective pressures or whether intersexual correlations

390 arise due to incomplete sexual differentiation of sexual behaviours [14,45]. Apart from
391 demonstrating a genetic basis for the trait in Coleopteran beetles [12-14], additional
392 information about the evolutionary genetics of SSB is derived almost exclusively from
393 studies of human homosexuality, in particular, male homosexuality [46-51]. Despite these
394 comparatively more extensive research efforts, SSB and sexual orientation are obviously not
395 homologous traits [3], and drawing direct parallels between such taxonomically distinct
396 species as human beings and fruit flies is unlikely to be of much value [15]. Nevertheless,
397 with increased research attention in other organisms it may eventually become feasible to
398 study the genetics of SSB using a comparative approach, which would enable researchers to
399 test the generality of evolutionary hypotheses for its maintenance.
400 It is debatable whether SSB represents a unified phenomenon across taxa or
401 whether its functions and evolutionary origins are too multifarious to be studied except in
402 the context of a single species or taxonomic group. Some broad themes are beginning to
403 emerge, with reviews of arthropods [16] and work on other invertebrates such as the deep
404 sea squid *Octopoteuthis deletron* [52] suggesting indiscriminate mate choice may underlie
405 SSB when mating opportunities are limited. In addition, studies in avian taxa have used the
406 comparative method to examine life history correlates of female-female pair bonding and
407 test phylogenetic signals underlying the expression of SSB [53], and primatologists have
408 studied SSB from a perspective more focused its role in social transactions in highly social
409 species [2]. These studies suggest different sources of selection maintain this apparently
410 non-adaptive trait with different indirect fitness benefits depending on a variety of
411 ecological and life history factors. To critically evaluate evolutionary hypotheses about the
412 origins and maintenance of SSB, more genetic research is clearly required across a broader
413 range of organisms.

Author Contributions. NWB and MGR designed experiments. JLH executed experiments and collected data. NWB and MGR performed statistical analyses. NWB, JLH and MGR wrote the manuscript.

Acknowledgements. We are grateful to Tanya Sneddon, Maureen Cunningham, David Forbes, Harry Hodge and Keith Haynes for technical assistance. This study was supported by Natural Environment Research Council (NERC) fellowships to NWB (NE/G014906/1, NE/L011255/1) and a NERC grant to NWB and MGR (NE/I016937/1).

DATA ACCESSIBILITY

Behavioural data unique to this study, plus fecundity data, is archived at Dryad (Dryad doi:10.5061/dryad.t0c3s). Additional DGRP phenotype data are archived at http://dgrp.gnets.ncsu.edu/.

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541 **TABLES**

542

543 **Table 1.** Predictions for overdominance and sexual antagonism models of SSB (adapted

544 from [16]) evaluated in the current study.

TRAITS	**PREDICTIONS***	
	overdominance	**sexual antagonism**
chromosomes	autosomal inheritance	strong X-linkage
dominance	dominance effects	no dominance effects
fecundity	heterozygote fitness advantage	male SSB correlated with female fitness
sex ratio	no sex ratio bias	male SSB correlated with female-biased sex ratio

545 * These and other genetic models for the maintenance of SSB are not necessarily mutually exclusive.

546 **Table 2.** General linear models of female fecundity in diallel crosses. (A) Comparison of all
547 crosses, examining differences between diagonal (inbred) crosses and off-diagonal
548 (outbred) crosses. (B) *Post-hoc* analysis examining variation among off-diagonal cross types
549 to assess whether [low-SSB x low-SSB] crosses show lower fecundity than the rest.

	(A) initial analysis including all crosses		
factor	**d.f.**	**F**	**P**
block	1	23.18	<0.001
inbreeding	1	96.33	<0.001
block x inbreeding	1	0.77	0.380
maternal genotype	3	11.45	<0.001
paternal genotype	3	9.52	<0.001
error	242		

550

	(B) *post-hoc* analysis excluding diagonal data		
factor	**d.f.**	**F**	**P**
block	1	75.17	<0.001
cross type	3	7.36	<0.001
block x cross type	3	1.86	0.138
error	205		

551

552 **Table 3.** General linear models of offspring sex ratio in diallel crosses. (A) Comparison of all
553 crosses, examining differences between diagonal (inbred) crosses and off-diagonal (outbred)
554 crosses. (B) *Post-hoc* analysis examining variation among off-diagonal crosses to assess
555 whether offspring sex ratio varied among cross types.

	(A) initial analysis including all crosses		
factor	**d.f.**	**F**	**P**
block	1	2.70	0.102
inbreeding	1	0.04	0.845
block x inbreeding	1	3.90	0.049
maternal genotype	3	0.36	0.781
paternal genotype	3	3.61	0.014
error	242		

556

	(B) *post-hoc* analysis excluding diagonal data		
factor	**d.f.**	**F**	**P**
block	1	0.38	0.538
cross type	3	2.27	0.081
block x cross type	3	0.56	0.644
error	205		

557

FIGURE LEGENDS

Figure 1. Original SSB screen compared to blind validation screen in 8 DGRP lines. Solid black lines indicate DGRP lines that expressed high SSB in the original screen, whereas dashed black lines indicate lines that expressed low SSB in the original screen. The intermediate-SSB line is shown in grey. The lowest line has been jittered to aid visualisation.

Figure 2. Variation in the expression of male SSB among focal lines. For lines that were re-tested in the blind validation procedure, the values indicate the combined incidence of SSB across both blocks. Lines are ordered on the x-axis according to their original numerical identifier.

Figure 3. SSB in male offspring from crosses between low-SSB and high-SSB parents. Paternal influences on the expression of SSB were stronger than maternal influences: offspring show SSB levels that resemble the trait value of their father's line more closely than that of their mother's line. Data from the appropriate within-line crosses (low,low or high,high) is included to allow comparison with maternal and paternal trait values. Note that data from each of the two (low,low) and two (high,high) crosses appear twice in the graph. We used two low-SSB and two high-SSB lines in crosses, so there were four possible combinations involving a pair of low and high lines. These are grouped along the horizontal rows, with the lines used indicated to the left. Shading in the circles indicates which parents were low-SSB or high-SSB.

Figure 4. (A) Fecundity of female offspring from diallel crosses. Cross type is indicated above the graph, circles indicate means and error bars indicate one standard error. The order of the cross is indicated as (mother,father). (B) Maternal and paternal effects on offspring sex ratio. Untransformed sex ratio data is shown, and the dashed line indicates a 1:1 offspring sex ratio. Circles indicate means and error bars show one standard error. Circle shading corresponds to parental genotypes. In both panels, overlapping data points were jittered to facilitate visualisation.