Limits to environmental masking of genetic quality in sexual signals

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Abstract

There is considerable debate over the value of male sexual ornaments as signals of genetic quality. Studies alternately report that environmental variation enhances or diminishes the genetic signal, or leads to crossover where genotypes perform well in one environment but poorly in another. A unified understanding is lacking. We conduct the first experimental test examining the dual effects of distinct low and high genetic quality (inbred versus crossed parental lines) and low, through high, to extreme environmental stress (larval diets) on a condition-dependent male ornament. We find that differences in genetic quality signalled by the ornament (male eyespan in *Diasemopsis meigenii* stalk-eyed flies) become visible and are amplified under high stress but are overwhelmed in extreme stress environments. Variance among distinct genetic lines increases with environmental stress in both genetic quality classes, but at a slower rate in high quality outcrossed flies. Individual genetic lines generally maintain their ranks across environments, except among high quality lines under low stress conditions, where low genetic variance precludes differentiation between ranks. Our results provide a conceptual advance, demonstrating a unified pattern for how genetic and environmental quality interact. They show when environmental conditions lead to the amplification of differences in signals of genetic quality and thereby enhance the potential indirect genetic benefits gained by female mate choice.

1. Introduction

Many exaggerated male sexual ornaments are thought to have evolved to provide information about the genetic quality of the signaller [1,2,3,4,5,6,7]. Yet these traits typically also respond strongly to environmental variation [8,9,10,11], and it is unclear what impact this has on their signalling function [12,13]. Does increasing environmental stress expose the underlying genetic differences in quality or mask them by overwhelming the genetic signal? Different studies have variously reported that environmental variation enhances [1,2,14,15] or diminishes [16,17,18] the phenotypic signal of genetic quality. Others reveal crossover, where genotypes that perform well in one environment do poorly in another [17,19,20,21]. These contrasting outcomes arise from a lack of consistency in experimental approach. The main problem is that analysis has focussed on genetic variation rather than distinct classes of genetic quality, coupled to a limited rather than wide range of environmental stress. We present a novel experimental design that addresses both of these deficits, which leads us to propose a unified understanding of how variation in genetic quality is impacted by environmental variation. This gives a far clearer understanding of the conditions under which sexual display traits can function to accurately reveal the genetic quality of signallers [22].

In this study, we adopt an integrated experimental approach, and for the first time examine the impact of a similar wide range in both genetic and environmental quality. We chose to focus specifically on male eyespan variation in the stalk-eyed fly [23,24] as this trait has been subject to extensive previous work. Male eyespan is highly exaggerated due to female choice [11,25,26,27,28,29,30,31,32] and also functions as a signal in male-male antagonistic interactions over mate mating sites [25,33,34]. It is

67 highly condition-dependent relative to other traits in relation to both genetic [24, but see
68 36] and environmental [9,11,35] stressors, and is responsive to a range of environmental
69 stress types [9,11,24,35,37,38], while genetically distinct families have also been shown
70 to respond differently to environmental stress [1].
71
72 Our novel experimental design to study genetic quality-by-environment (G x E)
73 interactions in signalling traits exploits pre-defined genetic [39,40,41] and environmental
74 quality classes. To vary genetic quality, crosses were made within or between a set of
75 parental inbred lines ($f \sim 0.908$ [42]; Figure 1). This allowed us to compare low genetic
76 quality, highly homozygous "incross" lines ($n = 16$, crosses = 67) with high genetic
77 quality, highly heterozygous "outcross" lines ($n = 17$, crosses = 50). We used incross and
78 outcross lines not to study the effect of inbreeding *per se*, but because previous work
79 unambiguously shows they correspond to low and high genetic quality classes
80 respectively [24]. The large number of independent crosses within or between lines
81 allows us to capture the contribution of genetic variation in the sexual ornament among
82 low and high genetic quality classes.
83
84 We likewise generated a wide range of environmental quality variation through
85 reductions in the amount of food available to developing larvae. This approach is a well-
86 established method for creating stress in holometabolous insects
87 [1,11,15,26,30,35,37,40,43,44,45] and has been used extensively in prior stalk-eyed fly
88 studies [9,11,24,30], where it generates body size variation equivalent to the range found
89 in natural populations [32,35]. Eggs were collected from each cross, and reared under
90 conditions of low, high and extreme environmental stress. Intermediate levels of stress
91 between low and high were omitted due logistical constraints (the study analysed 1185

 # Figure 1.

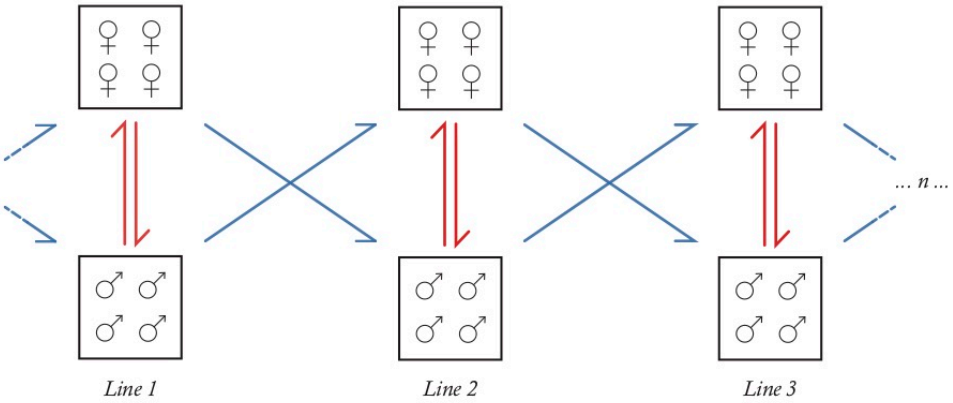

Fig. 1. The crossing protocol used to generate incross and outcross offspring. Each inbred line was crossed with itself to create incross flies (red), or with another inbred line to create outcross flies (blue). Each cross used 4 males and 4 females and was repeated to generate two families per cross. #

117 flies from 117 experimental crosses), and because those stress levels have been
118 extensively investigated previously [11]. The extreme level was defined as the least food
119 level where larval viability was not seriously impaired (see below)
120
121 Our use of the terminology low/high genetic quality and low/high/extreme
122 environmental quality is necessarily arbitrary but justified in terms of the experimental
123 design and in the results that follow. We comment further on these definitions in the
124 Discussion. The innovation in our experimental design lies in delivering controlled
125 manipulation of genetic quality *and* environmental stress, over several pre-defined
126 quality levels, thereby enabling an in depth exploration of genetic quality-by-
127 environment (G x E) variation in a male sexual ornament. We use this to investigate the
128 signalling utility of the male ornament in providing information about indirect genetic
129 benefits through female mate choice.
130

131 ## 2. Material and methods

132

133 **(a) Production of experimental flies**

134 A suite of 105 inbred lines were founded from a stock of *Diasemopsis meigenii* [24], an
135 African stalk-eyed fly, derived from flies collected in South Africa by S. Hilger in 2000.
136 After 11 generations of full-sib mating, the extant lines were bred in cage culture (~200
137 flies/cage). For this study, 17 lines that varied between generations F_{24}–F_{33} were chosen
138 as parental lines to use in crosses, with flies collected as eggs laid on petri dishes
139 containing excess puréed sweet corn (10g). At eclosion, flies were placed in large cages
140 (15l), separated by sex (~2 weeks) and raised until sexual maturity (~10 weeks). Adults
141 were fed puréed sweet corn with antifungal Nipagin, replenished twice per week. All

142 flies were maintained using our standard protocol at 25°C on a 12:12 hour light:dark
143 cycle, with fifteen-minute artificial "dawn" and "dusk" periods (reduced illumination) at
144 the start and end of the light phase throughout the experiment.
145
146 **(b) Variation in genetic and environmental effects**
147 Variation in genetic quality was achieved using previously created inbred lines [24] in a
148 crossing protocol (Figure 1; modified from [36] and [24]). "Incross" flies were created
149 from male-female crosses within an inbred line and "outcross" flies were created from
150 crosses between different inbred lines. In each cross, 4 adult males from line x and 4
151 adult females from line y were allowed to mate in a 1.5l pot ($x = y$ for incross, $x \neq y$ for
152 outcross). Reciprocal male x - female y and female x -male y pots were set up. Multiple
153 replicates of each cross (between 1-8) were set up, with higher rates for inbred crosses as
154 they were less fecund. Eggs were collected twice weekly over 23 days. In all, 142 crosses
155 were set up, of which 117 generated sufficient offspring across the food treatments: 67
156 incrosses of 15 inbred lines and 50 outcrosses between 16 pairs of inbred lines. An inbred
157 line was used in an incross the same number of times as it was used in an outcross, and
158 as far as possible equal numbers of live adult males and females were collected from each
159 line, to balance sex chromosomal, cytoplasmic and other male/female parental effects.
160
161 Incross flies have low genetic quality as they are highly homozygous, being derived from
162 inbred lines created by repeated brother-sister pairings (11 generations), with an expected
163 inbreeding coefficient of $f \sim 0.908$ [24]. In contrast, outcross flies have high genetic
164 quality as they are expected to be heterozygous for most of the alleles fixed in the
165 parental inbred lines from which they are derived. Although the terms – low and high
166 genetic quality – are arbitrary, there was evidence of substantial heterosis in a variety of

167 traits when inbred flies were crossed, so the terms reflect the nature of these two genetic
168 groups [24].
169
170 For each incross or outcross, fertilised eggs were placed in groups of 5 in petri dishes
171 containing two cotton pads, 15ml water and 5ml of food medium. Three qualities of
172 food medium were used with "pure" corn diluted with water at ratios of 1:1, 1:10, and
173 1:20, which we designate as "low", "high" and "extreme" stress respectively. "Pure"
174 corn was made by forcing puréed sweet corn kernels through a fine sieve to remove
175 husks and provide homogeneity. Food qualities were chosen based on a pilot study, with
176 levels of food stress used that were found to lie within normal rates of egg-adult survival
177 (Figure 2, see SI.C). Although the terms – low, high and extreme environmental stress –
178 are again arbitrary, they nonetheless capture particular qualities. The low food level was
179 similar to the standard media on which larvae are raised. The high food level was used
180 previously where it was associated with reduced size in a variety of traits [24]. The
181 extreme food level constitutes the far end of the stress spectrum before differential
182 survival is evident (Figure 2). In the range used (i.e. 1:1 to 1:20), egg-to-adult survival did
183 not differ in the pilot, and was at ~50%. We did not go beyond this level, as a serious
184 loss of adults would have placed greater logistical difficulties in delivering the already
185 considerable sample size in the experiment. In the main experiment, a census of pupae
186 was additionally made as a measure of survival for each cross in each environment.
187
188 **(c) Adult morphology**
189 After eclosion, flies of each cross were collected and frozen at -20°C. All males were
190 measured for eyespan (the distance between the outermost tips of the eyes [9,46]) and
191 thorax (the distance between the centre of the most posterior point of the head to the joint

 # Figure 2.

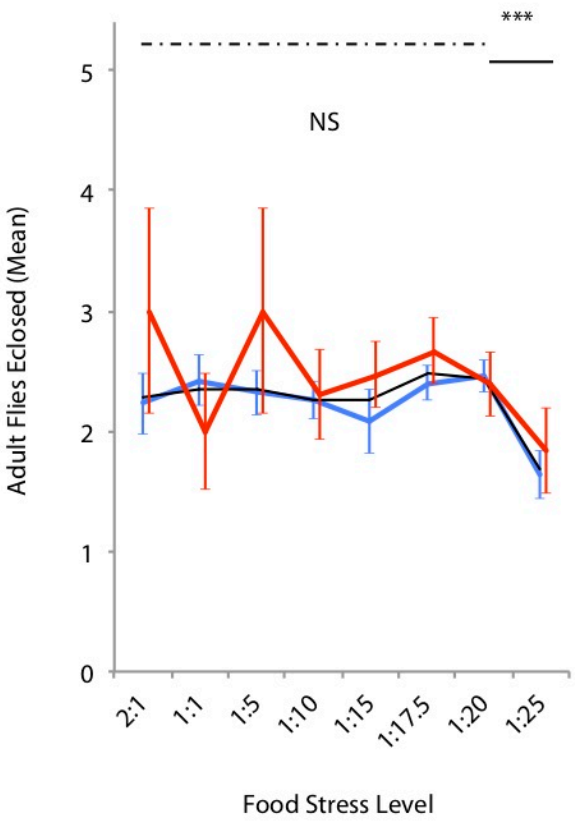

Fig. 2. Mean number of adult flies eclosing per petri dish (± SE) given seeding with five eggs, when subject to different larval treatments (ratio of corn:water), for inbred lines (red) and stock (blue), or when pooled (black). Pairwise comparison of adjacent treatments showed a significant drop in survival between the adjacent 1:20 and 1:25 treatments (solid line, *** $P < 0.001$), and no difference between other adjacent levels (dashed line, NS). A similar pattern was observed for inbred and stock considered separately across the adjacent 1:20 and 1:25 treatments (both $P < 0.001$). Inbred and stock populations did not differ at any food level (all $P > 0.05$). Data is based on a pilot experiment (17 crosses, 10 stock, 7 inbred, $N = 218$ stock, 68 inbred; details SI.C). #

between the meta-thoracic legs and the thorax [47,48]) to a tolerance of 0.01mm, using a video camera mounted on a monocular microscope and ImageJ image capture software v.1.46 [49]. The repeatability of these morphological trait measurements is very high at >99% [9]. In total 1186 males were phenotyped. All measurements were made blind by JMH. In a few cases (n = 9), a measurement was not included in the dataset due to sample damage.

(d) Statistical analysis

To test for effects of incross/outcross genetic quality (G), environmental (E) and the G x E interaction on morphological trait variation, several general linear mixed effects models (GLMMs) were fitted via REML. In each model, G, E and their interaction were included as fixed effects. Male parental line and female parental line were included as random effects, as was cross and its interaction with E. Additional random effects of male line x E, male line x G, female line x E and female line x G explained zero variance and so were removed in model simplification. GLMMs for male eyespan had thorax added as a covariate to control for body size. Thorax length accounted for a significant portion of variance, but its addition did not substantially alter the results (for completeness, analyses of absolute trait values are given in the SI.A). GLMM models fitted pairwise to low versus high and high versus extreme environmental stress were used to further investigate the basis of the observed G x E patterns, as finally were two-tailed t-tests at each level of E to test whether incross male eyespan was larger or smaller than outcross male eyespan.

Coefficients of variation (CVs), the ratio of the standard deviation to the mean, were used to assess how variance in male eyespan responded to genetic quality,

242 environmental and G x E stress. CVs control for changes in variance purely as a function
243 of size, and are considered to be less biased than heritability estimates in G x E studies
244 [50]. Least square means for male relative eyespan were extracted from GLMMs for
245 each cross, for each E and G, to calculate among-cross CVs. Among-cross CVs were
246 then compared between incross and outcross using modified signed-likelihood ratio tests
247 (M-SLRT; [51]) in each environment, and also across environments (L-H-X), both
248 overall and for incross and outcross. Finally, adjacent environment pairs were contrasted
249 for among-cross CV, low with high (L-H) and high with extreme (H-X), for each genetic
250 quality. The among-cross contrasts were conducted in the R-package 'cvequality' [52].
251
252 To explore the consistency of genetic lines across environments, another key aspect of G
253 x E interactions, genetic correlations (r_g) across adjacent environments were calculated.
254 GLMMs were fitted with cross as a random effect and the variance component for cross
255 was extracted for each environment. GLMMs were then carried out between pairs of
256 adjacent environments (L-H, H-X), with the cross x E interaction included as a random
257 effect, and the interaction variance component extracted. As before, thorax length was
258 added as a fixed covariate to control for body size. An estimate of r_g was then calculated
259 as:
260
261 $r_g = \sigma_{1,2} / \sqrt{\sigma^2_{1,1}\ \sigma^2_{2,2}}$,
262
263 where $\sigma^2_{1,1}$ and $\sigma^2_{2,2}$ are the genetic variances in environments 1 and 2 respectively, and
264 $\sigma_{1,2}$ is the genetic covariance between the two environments [53]. Broad bounds of the r_g
265 values were tested via model simplification and likelihood ratio tests (details in SI.A)
266

(e) Statistical software used

All statistical analyses were conducted in JMP v.12.0.1 (SAS Institute 1989-2015) and R v.3.4.2 [54]. GLMM tables, effect coefficients and extended methods are shown in SI.A.

3. Results

(a) Response in mean trait

As expected, male eyespan ($F_{2,45.47}$ = 693.4, P < 0.001) and thorax ($F_{2,41.80}$ = 343.4 P < 0.001) were smaller under higher environmental stress. The same was the case under genetic stress for eyespan ($F_{1,22.94}$ = 4.783, P = 0.028) but not for thorax, though its response was in the same direction ($F_{1,13.78}$ = 3.222, P = 0.095). After controlling for body size variation, the same direction of change was observed in male eyespan for environmental ($F_{2,54.66}$ = 258.1, P < 0.001) and genetic stress ($F_{1,7.421}$ = 6.203, P = 0.039). All following comparisons report relative trait values.

In addition, there was a genetic quality-by-environment interaction ($F_{2,39.33}$ = 5.379, P = 0.009, Figure 3a). The nature of the G x E was evident from comparison of adjacent environments. The difference in male eyespan between incross flies with low genetic quality and outcross flies with high genetic quality increased from low to high environmental stress (i.e. scale variance G x E, $F_{1,18.35}$ = 6.352, P = 0.021). But there was convergence between genetic quality classes after a further increase from high to extreme environmental stress (i.e. inverse scale variance G x E, $F_{1,15.64}$ = 8.664, P = 0.010). This pattern was confirmed by looking at environments separately. The difference between incross and outcross male eyespan was evident at high (t = 8.65, df = 19.81, P < 0.001),

 # Figure 3.

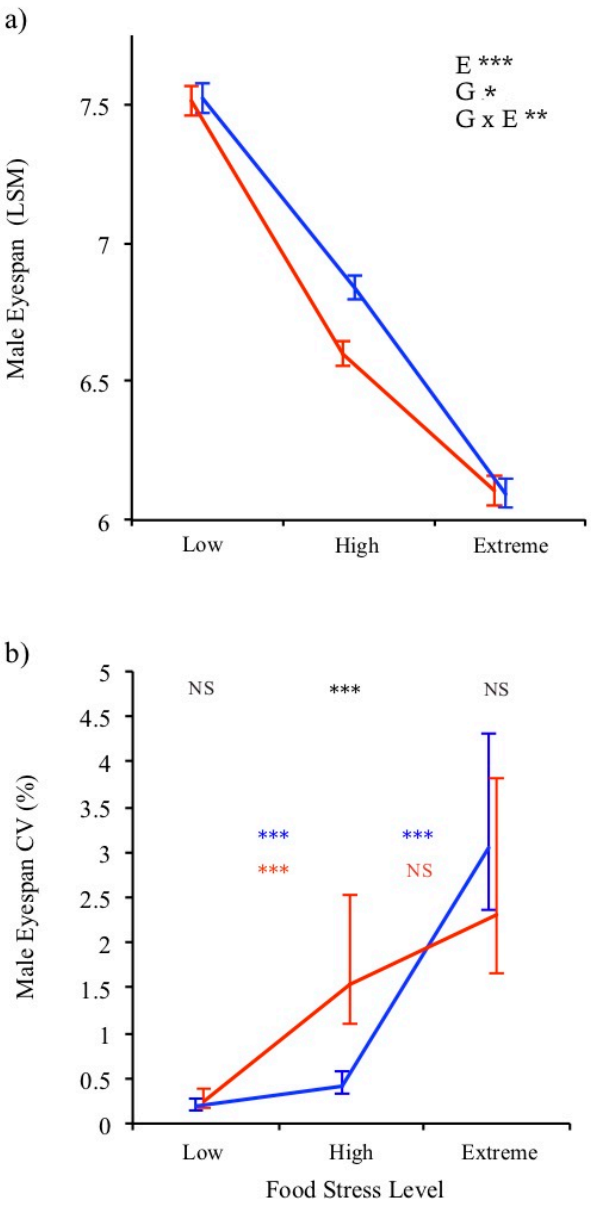

Fig. 3. a) Male eyespan (least-squares mean ± SE) and b) coefficient of variation (CV ± 95% CI) across environmental stress (low, high and extreme) and genetic class, incross (red) and outcross (blue). The red and blue lines are shown for illustrative purposes and clarity. Asterisks denote significance: NS non-significant, * < 0.05, ** $P < 0.01$, *** $P < 0.001$. For CVs, the significance of incross versus outcross contrasts are displayed above each food level category (black asterisk at the top). The significance of within incross (red asterisks) and outcross (blue asterisks) contrasts are shown between pairs of adjacent food levels. Incross and outcross lines are jittered for clarity. #

317 but absent at low (t = 1.98, df = 19.79, P = 0.073) and extreme levels of environmental
318 stress (t = -1.01, df = 21.87, P = 0.298).
319
320 When comparisons were limited to incross lines, there were environmental (χ_1^2 = 276.7,
321 P < 0.001) and genetic line differences (χ_1^2 = 11.08, P < 0.001) but no G x E interaction
322 (χ_1^2 = 4.281, P = 0.509). A similar pattern was found in outcross lines, where there were
323 environmental (χ_1^2 = 243.4, P < 0.001) and genetic line differences (χ_1^2 = 5.14, P = 0.023)
324 but no G x E interaction (χ_1^2 = 7.71, P = 0.173). These results indicate that G x E
325 interactions were only apparent in the comparison of genetic quality (i.e. incross vs.
326 outcross), and not in the comparison of genetic lines within low or high genetic quality
327 groups.
328
329 **(b) Response in trait variance**
330
331 The genetic quality G x E pattern was further examined by looking at the among-cross
332 variance in the response to stress. Coefficients of variation (CV) were used to control for
333 the positive scaling in variance due to changes in mean trait size. Male eyespan among-
334 cross CV (Figure 3b) was larger with greater environmental stress overall (R_M = 26.55, P
335 < 0.001), and separately for incross (incross R_M = 40.00, p < 0.001) and outcross lines
336 (R_M = 130.35, P < 0.001). But the extent of increase in CV from low to high
337 environmental stress was considerably more marked among incross males with low
338 genetic quality (1.30% increase, R_M = 28.95, P < 0.001) than outcross males with high
339 genetic quality (0.23% increase, R_M = 11.95, P < 0.001). Differences among outcross lines
340 were revealed to a much greater extent once the level of environmental stress increased
341 even further, in the transition from high to extreme environmental stress (2.63%

increase, $R_M = 57.34$, $P < 0.001$). This pattern contrasted again with males from incross lines, where CV did not differ between high and extreme environmental stress levels (0.78% increase, $R_M = 1.848$, $P = 0.174$, Figure 3b). As for mean eyespan, the difference between incross and outcross CV was seen only under high environmental stress (low stress $R_M = 0.814$, $P = 0.367$, high stress $R_M = 24.32$, $P < 0.001$, extreme stress $R_M = 1.148$, $P = 0.284$; Figure 3b).

(c) Across environment genetic correlations

To further evaluate the role of male eyespan as a signal of genetic quality, we examined whether genetic lines performing well in one environment performed well across all environments (Figure 4), a critical part of the G x E pattern. When pooling all lines, there was a positive genetic correlation (r_g) between low and high ($r_g = 0.563$, $\chi^2_1 = 11.54$, $P < 0.001$), and high and extreme environmental stress ($r_g = 0.360$, $\chi^2_1 = 22.94$, $P < 0.001$). There was also a genetic correlation-by-environment interaction between low and high stress ($\chi^2_1 = 15.27$, $P < 0.001$) in which the genetic lines fanned out under higher environmental stress.

Analysing the two genetic quality classes separately, for low quality incross lines, genetic correlations (r_g) were positive between low and high ($r_g = 0.267$, $\chi^2_1 = 4.184$, $P = 0.041$), as well as between high and extreme stress environments ($r_g = 0.082$, $\chi^2_1 = 11.11$, $P < 0.001$). For the high quality outcross lines, there was no genetic correlation between low and high stress environments ($\chi^2_1 = 0.221$, $P = 0.469$), but r_g was positive between high and extreme stress environments ($r_g = 0.171$, $\chi^2_1 = 5.189$, $P = 0.023$). The lack of r_g was due to severely reduced variation among outcross lines in the low ($CV_{low} = 0.188$, CV_{high}

 # Figure 4.

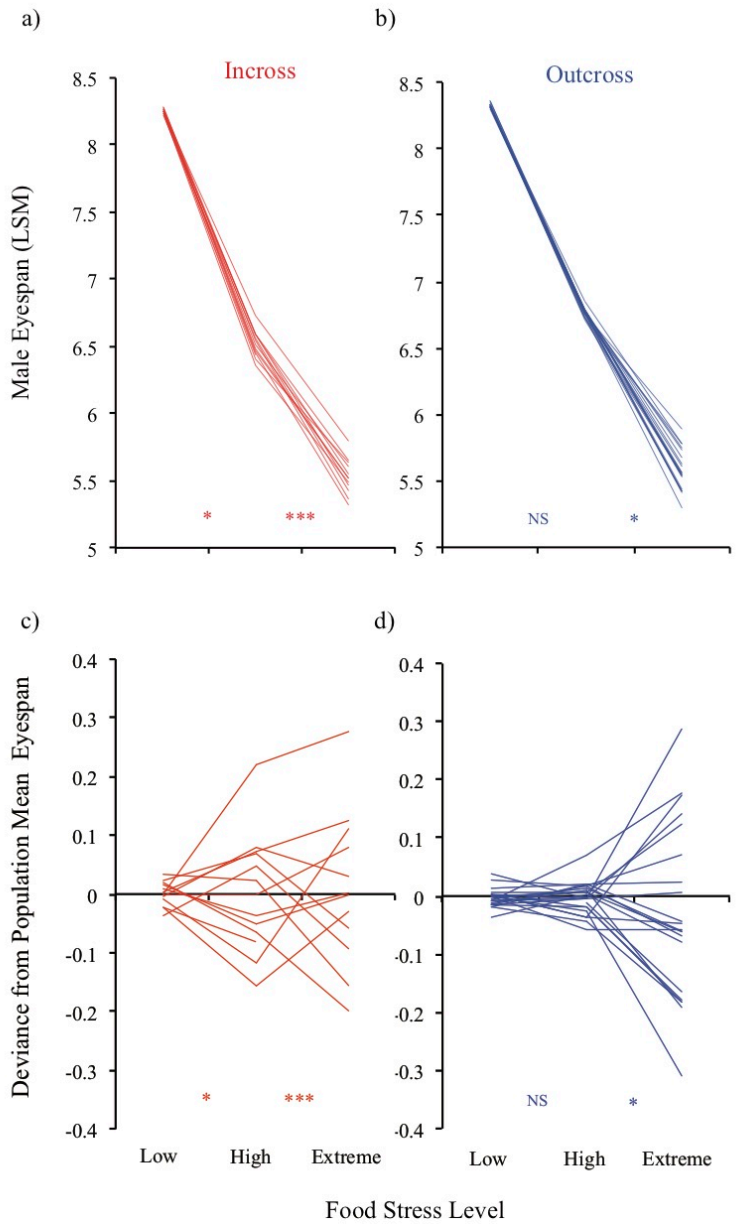

Fig. 4. Mean male eyespan (least-squares mean relative values) at each environmental stress for each cross a) incross (red) and b) outcross lines (blue). Asterisks denote significance of the effect of cross, NS non-significant, * $P < 0.05$, *** $P < 0.001$. An alternative representation is shown as the absolute deviation of each line from the c) incross and d) outcross population mean. Error bars are excluded for clarity. #

392 = 0.416, $CV_{extreme}$ = 3.05) compared to high (R_M = 57.34, $P < 0.001$) or extreme stress
393 environments (R_M = 88.17, $P < 0.001$; Figure 4).
394
395 **(d) Survival across G and E stress**
396 Larval survival was measured through a census of pupae. There was a survival effect of
397 E ($F_{1,64.36}$ = 64.36, $P < 0.001$) but not of G ($F_{1,20.04}$ = 0.852, P = 0.367) or G x E ($F_{2,64.28}$ =
398 0.976, P = 0.382). The effect was a reduction in survival at extreme environmental stress
399 (pupae counts: LSM ± SE low = 2.17 ± 0.10, high = 2.29 ± 0.09, extreme = 1.55 ± 0.09).
400 A Tukey's HSD test confirmed that survival was lower under extreme relative to either
401 low or high environmental stress level ($P > 0.05$). Survival did not differ between incross
402 and outcross in any of these comparisons (all $P > 0.05$, see SI.A).
403
404
405 ## 4. Discussion
406
407 In this study we explicitly test whether environmental stress amplifies or obscures the
408 signal of genetic quality in male sexual ornaments. We do so in a unique way by direct
409 manipulation of *both* genetic quality and environmental stress, the latter over multiple
410 levels. The results enable us to put forward a unified explanation of how genetic and
411 environmental quality interact, advancing our understanding of the genetic benefits of
412 mate choice, with the potential to explain the diverse responses seen in other systems.
413
414 The response of male eyespan – the primary sexual ornament in *D. meigenii* – accords
415 with previous studies in stalk-eyed flies, showing that this male ornament is a sensitive
416 signal of both environmental [9,11,35] and genetic stress [1,24]. Of greater interest, the

417 new data captures a full range of G x E interactions. The difference between low and
418 high genetic quality, in both eyespan mean and variance (coefficient of variation),
419 increases with the transition from low to high environmental food stress (Figure 3). This
420 is an example of "scale variance" G x E in which higher environmental stress amplifies
421 genetic differences. It has been observed across a range of species, for example in
422 structural wing pigmentation (UV angular visibility) in the butterfly *Colias eurytheme* [55],
423 male song attractiveness in the lesser waxmoth, *Achroia grisella* [2], and attractiveness
424 traits in the black scavenger fly, *Sepsis punctum* [15], all examples of traits associated with
425 sexual success. In contrast, the difference between our low and high genetic quality
426 classes, in both eyespan mean and variance, decreases with the transition from high to
427 extreme environmental food stress (Figure 3). This reversed pattern is an example of
428 "inverse scale variance" G x E in which stress denudes genetic differences. It again has
429 been observed across a range of species, for example, iridescent and orange area in the
430 guppy *Poecilia reticulata* [17], cuticular hydrocarbon blend in *Drosophila simulans* [16], and
431 to a more limited extent, UV brightness in *C. eurytheme* [55].
432
433 Our results are novel and striking because we see *both* scale variance and inverse scale
434 variance in the same trait in a single species. This leads us to propose a unified
435 hypothesis for G x E interactions in signals of quality. Moderate to large increases in
436 environmental stress lead to amplification of the phenotypic expression of genetic
437 quality, whereas as environmental stress becomes extreme, increases in phenotypic
438 variation overwhelm the underlying genetic differences in quality. We note that in some
439 previous studies, separate traits respond differently to environmental stress, suggesting
440 variation in the threshold at which amplification transitions to restriction (e.g. [2, 55]).

441 Future studies will be needed to identify which characteristics are associated with
442 sensitivity levels in different traits, and whether these relate to costs of trait expression.
443
444 Yet, some evidence from other studies of sexual ornaments seems to contradict the
445 unified hypothesis which report no interaction between genetic and environmental stress,
446 for example in morphological traits and cuticular hydrocarbons in *D. melanogaster* [40]
447 and several sexual traits in *P. reticulata* guppies [39]. Both of these experiments examined
448 groups that differ predictably in genetic quality (hemiclonal lines and inbred versus
449 outbred lines, respectively). But the lack of response likely reflects the application of
450 insufficiently intrusive environmental stress. For example, the "stressful" environment in
451 guppies was a moderate density [39], while that in *D. melanogaster* was a minor reduction
452 to 70% of the normal diet [40]. A previous study in stalk-eyed flies likewise found little
453 impact of food reduction of this order [11]. For comparison, our dilution for extreme
454 stress was a restriction to just 5% of the standard diet. Moreover, as each of these studies
455 used just two levels of environmental stress, analysis of complex G x E was precluded.
456 This is not a criticism of either study, which had different goals to ours, but highlights
457 that neither would provide an adequate test of our hypothesis. Another commonly
458 reported pattern across diverse species, also potentially at odds with our interpretation, is
459 "crossover" G x E in which different genetic lines are superior in different environments.
460 This has been shown for male signal rate in the lesser waxmoth [19] and song traits of
461 *Enchenopia* treehoppers [20]. However, "crossover" G x E is not really a distinct
462 category, and can co-occur with "scale" or "inverse scale" G x E patterns [56]. For
463 instance, crossover embedded within G x E scale variance patterns in the lesser
464 waxmoth [2] and inverse scale variance in the guppy [17] has been observed. Once
465 again, the interpretation that crossover dominates the G x E pattern requires

466 investigation of sufficient levels of environmental stress relative to the traits in question.
467 Without this, crossover should only be seen as part of G x E response, exerting
468 ambiguous limits on the signalling function of the sexual ornament.
469
470 It is vitality important to examine a range of environments from low through to an
471 extreme form of stress, alongside similar dimensions of genetic quality variation. Distinct
472 classes of environmental quality variation were created in a standard manner through
473 food restriction applied to developing larvae [1,11,15,30,40,45]. These treatments
474 differed from previous studies in the use of food dilution to an "extreme", defined as the
475 point before larval survival showed a clear-cut decline (Figure 2). The reason for
476 choosing this point was in part logistical, in order to easily collect similar sample size
477 across the different stress levels. We also wanted to avoid the possibility that differential
478 survival causes changes in trait mean and variation across the different genetic quality
479 and environmental stresses. Despite this precaution, there was a moderate effect of the
480 extreme environmental stress on larval survival. This could have contributed to the trait
481 patterns observed if there was a lower level cut-off in the eyespan of survivors. We
482 suspect this effect was minor as the mean was lowest and the CV highest in the extreme
483 environment (Fig. 3), and more importantly, the survival deficit was equal across incross
484 and outcross flies. Our conclusions appear to be robust. Our use of food quantity as an
485 environmental stress was for its ease of manipulation and its use in many previous
486 studies. Competition for food is likely to be a factor in many species and so we suspect
487 that the results we report here are general stress responses. This needs to be established
488 through comparison with other stresses, such as fluctuations in temperature, pH or food
489 quality, that are part of the normal range of environmental stress in the wild [57].
490

To create distinct classes of genetic quality, a set of highly homozygous inbred lines (incross) were compared against crosses between lines (outcross) which are predicted to be highly heterozygous for the mutational load carried by incross lines. In the pilot experiment (Fig. 2), as well as the actual experiment, there was no difference in egg-to-adult survival between flies in the incross and outcross genetic quality treatments. The lack of a viability difference suggests that there was a strong purging of deleterious alleles during the creation of the inbred lines, as is expected and observed in other studies [58-60]. Our objective was not to study the inbreeding *per se*, as this is unlikely to be the object of female mate preference in this species. Rather we use inbreeding status as an investigative tool, in order to uncover the full nature of genetic quality-by-environment interactions on variation in signal trait size. In particular, previous G x E studies have failed to use a sufficient range of variation in genetic quality. A typical approach is to use distinct genetic lines, like brother-sister families [1,20] or inbred lines [2]. But groups that differ predictably in genetic quality have not been examined properly against a wide range of environmental stress [39-40]. Independent lines provide information about genetic variation but may differ only slightly, and unpredictably, in genetic quality, and then only with differences established *post hoc*. In our study, we distinguish between variation in genetic *quality* in the comparison of incross and outcross flies, and genetic variation between lines within these quality categories. In accordance with prior studies [1], our results show differences in performance between lines. Crucially, there was no among-line G x E once analysis was limited to a particular genetic quality class, both for incross and outcross. The set of lines in each genetic quality class appear to have been sufficiently similar in quality that they responded in an equivalent manner when challenged with our wide range of environmental stress levels (Figure 4). Only the comparison between incross and outcross flies revealed a strong G x E interaction, in

516 which high quality (outcross) line resisted the effect of high but not extreme
517 environmental stress.
518
519 Taking the results together allows us to comment on sexual selection on males and the
520 potential indirect genetic benefits that arise from female mate choice. We expect sexual
521 selection to be severely attenuated under benign and extreme environmental stress, but
522 strong in high stress environments which amplify genetic quality differences. As stress is
523 likely to be the norm under common ecological conditions in nature, sexual selection
524 could often be stronger than currently estimated from laboratory experiments – typically
525 carried out under low stress conditions of *ad libitum* food, constant temperature, no
526 predators and parasites, and no ecological competitors. We note that our
527 experimentation used stress from a unimodal environment variable (food availability),
528 controlling all other physical and biotic factors, and that we used a simple measure of
529 male signalling, leaving aside other, more subtle aspects of male behaviour used in
530 female evaluation of their partners [46]. This implies that benign environmental
531 conditions, equivalent to low stress in our experiment (i.e. in which larvae have excess
532 food and little competition), are rare. Extreme environments are likewise also likely to be
533 rare as they are not those that maintain viable populations. The majority of
534 environments probably lie between the low and high regimes, which is consistent with
535 the considerable range in eyespan observed among wild caught stalk-eyed flies [32].
536
537 The outcome in nature for female choice will depend on the distribution of
538 environmental stress, its spatial and temporal variability, and hence its consequence for
539 the pool of available mates in a given population [12]. If conditions can be categorised as
540 low, high or extreme, then the indirect benefits of mate choice will be greatest in high

541 stress environments, as these bring out genetic differences to the greatest extent. As
542 genetic line correlations across environments were positive (with the exception of
543 outcross lines between low and high food stress, where a lack of variation precluded
544 reliable calculation), genetic differences will be evident to some extent in all
545 environments. Where environmental conditions in a population are a mixture of low,
546 high and extreme, individuals with the most exaggerated sexual ornaments will be an
547 assortment of those with high genetic quality from a range of environments diluted by
548 those less well genetically endowed but who experienced lower environmental stress
549 during development. This cuts at the indirect genetic benefits but nonetheless there will
550 be advantage to female mate choice. To conclude, while environmental variation places
551 contingencies on signalling, sometimes amplifying and sometimes muting its value,
552 genetic variation in quality between individuals will always to some extent be evident in
553 the sexual ornament and feed through to their offspring.
554
555

Data accessibility. Data are made available at the Dryad Digital Repository [TO ADD].

Author contributions. JMH, KF and AP conceptualised the study and methodology, and wrote, reviewed and edited the paper. The formal analysis was carried out by JMH, who with HACD carried out the experiments. Stalk-eyed fly resources were provided by AP and KF, who secured funding and supervised the project.

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SUPPLEMENTAL INFORMATION

Supplemental information includes all details of statistical effect size estimates for the tests of mean effects, and additional method details. [Available after formal publication.]

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