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Covariance among premating, post-copulatory and viability fitness components in *Drosophila melanogaster* and their influence on paternity measurement

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Abstract

In polyandrous mating systems, male fitness depends on success in premating, post-copulatory and offspring viability episodes of selection. We tracked male success across all of these episodes simultaneously, using transgenic *Drosophila melanogaster* with ubiquitously expressed green fluorescent protein (i.e. GFP) in a series of competitive and noncompetitive matings. This approach permitted us to track paternity-specific viability over all life stages and to distinguish true competitive fertilization success from differential early offspring viability. Relationships between episodes of selection were generally not present when paternity was measured in eggs; however, positive correlations between sperm competitive success and offspring viability became significant when paternity was measured in adult offspring. Additionally, we found a significant male × female interaction on hatching success and a lack of repeatability of offspring viability across a focal male's matings, which may underlay the limited number of correlations found between episodes of selection.

Introduction

Natural and sexual selection shape male traits that influence male mating success (Andersson, 1994), competitive fertilization success (Birkhead & Møller, 1998) and the ability to produce high-quality offspring (Roff, 2002). Consequently, premating sexual selection, post-copulatory sexual selection and offspring viability selection constitute three, potentially discrete, or interacting, episodes of selection that determine reproductive success. How selection shapes male phenotypes will depend on trait covariance and the relationships between traits and fitness across episodes (Møller & Alatalo, 1999; Neff & Pitcher, 2005).

Investigations to date reveal that fitness relationships across selection episodes may vary depending upon the nature of the traits and the study organism. For instance, males that are preferred by females or that otherwise experience relatively high mating success have been shown to further benefit from disproportionately high competitive fertilization success in the fruit flies *Drosophila simulans* (Hosken *et al.*, 2008) and *D. melanogaster* (Bretman *et al.*, 2009; Fricke *et al.*, 2010), the red flour beetle *Tribolium castaneum* (Lewis & Austad, 1994) and the guppy *Poecilia reticulata* (Evans *et al.*, 2003; Pilastro *et al.*, 2004; Locatello *et al.*, 2006; but see Evans, 2010). Similarly, males that excel in either episode of sexual selection have been demonstrated to produce offspring of superior quality in *D. melanogaster* (Taylor *et al.*, 1987; Gilchrist & Partridge, 1997) and *P. reticulata* (Evans *et al.*, 2004), as well as in the yellow dung fly *Scathophaga stercoraria* (Hosken *et al.*, 2003), the field cricket *Gryllus bimaculatus* (Wedell & Tregenza, 1999), the house cricket *Acheta domesticus* (Head *et al.*, 2005) and the marsupial *Antechinus stuartii* (Fisher *et al.*, 2006). These relationships are consistent with the 'good genes' hypothesis (reviewed in Andersson, 1994) or, in the case of relationships with fertilization success, the 'good sperm' hypothesis (Yasui, 1997). The latter model suggests that overall male condition determines sperm competitive success, resulting in higher condition males achieving both increased post-copulatory success and production of superior quality offspring. Conversely, males that are better at obtaining copulations have been found to be disadvantaged in competing for fertilization in the water strider

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Gerris lacustris (Danielsson, 2001) and to have less competitive ejaculates in one study of *P. reticulata* (Evans, 2010). Likewise, a male's success in sexual selection can be at odds with offspring reproductive success (e.g. *D. melanogaster*; Pischedda & Chippindale, 2006) and offspring viability selection (e.g. seed beetle *Callosobruchus maculates*; Bilde *et al.*, 2009), these patterns being attributable to sexual conflict.

Our understanding of the relationships among episodes of selection is further complicated by methodological challenges inherent in avoiding confounding offspring viability effects when assessing fertilization success. For example, competitive fertilization success is frequently measured as the proportion of adult progeny sired by a given male in a competitive mating. Differential genetic or parental effects on early offspring viability are known to exist (e.g. Gilchrist & Partridge, 1997; Barber *et al.*, 2001; Wedekind *et al.*, 2001; Evans *et al.*, 2007; García-González & Simmons, 2007, 2011) and can confound estimates of sperm competitive success (Gilchrist & Partridge, 1997; García-González, 2008). Parsing out the effects of these selective forces is largely constrained by the inability to nondestructively assay the paternity of eggs, which is a more accurate representation of competitive fertilization success.

Here, we present two experiments designed to discern the relationships among episodes of selection as well as to identify male and female influences on offspring quality. In the first experiment, we evaluated male and female influences on variance in offspring quality through noncompetitive isoline crosses. In a second experiment, we evaluated the performance of individual male *D. melanogaster* across all three episodes by mating each focal male to multiple females from a standard genetic background, while competing against a genetically standardized competitor male. We overcame the technical constraint of discriminating episode-specific fitness by using standard competitor males from a transgenic line of *D. melanogaster* expressing a ubiquitous green fluorescent protein marker (hereafter Ub-GFP). Ubiquitin is expressed in all *D. melanogaster* tissues at all life stages (Handler & Harrell, 1999), enabling us to track offspring paternity in competitive matings from egg to adult.

Materials and methods

Experiment 1: male and female influence on offspring viability

In order to establish male and female effects on offspring viability, noncompetitive crosses were conducted for flies from a total of twelve isolines (i.e. six isolines per gender). Eleven lines of experimental flies originated from a genetically variable, outbred laboratory stock (LHm) maintained with overlapping generations in population cages with > 1000 individuals on standard cornmeal–molasses–agar medium supplemented with yeast. The isolines were generated by first backcrossing fluorescent markers into the LHm population. Flies were then subjected to six generations of full-sibling matings, where founders for each isoline were randomly selected from the RFP- or GFP-marked LHm base populations. Six female-source isolines carried a sperm-specific RFP marker (protamine-RFP, Manier *et al.*, 2010), whereas five experimental male-source isolines carried the Ub-GFP marker and a sperm-specific GFP marker (protamine-GFP, Manier *et al.*, 2010). The Ub-GFP transgenic line was created by germline transformation of the pB[PUbnlsEGFP] vector using methods described in the study by Manier *et al.* (2010). The pB[PUbnlsEGFP] and pBΔSac helper plasmids were kindly provided by A. Handler (USDA-Agricultural Research Service, Gainesville, FL, USA; Handler & Harrell, 1999). A sixth male line carrying a ubiquitously expressed RFP marker (Ub-RFP) was created by germline transformation of w^{1118} flies, with P-element insertions and a w;Sco/CyO balancer (T. Van and J. Lipsick, unpublished), and was generously provided by the Lipsick laboratory (Stanford University, Stanford, CA, USA). All flies were reared at low density, achieved by moving parental pairs to new vials daily. All flies were collected shortly after eclosion under CO_2 anesthetization and housed by gender at a density of 20 flies per vial until mating. Flies were 2–4 days old for initial matings.

Each male was mated once to a nonexperimental LHm virgin female on the day preceding the experiment to avoid any effects related to male virgin status (e.g. Bjork *et al.*, 2007); these females were discarded after mating. Isoline crosses were replicated up to four times with a total of 124 successful matings across all lines with copulation duration quantified to the nearest minute. Following mating, each female was transferred by aspiration to a new oviposition vial with standard cornmeal–molasses–agar medium supplemented with live yeast once a day for 2 days.

Eggs in each vial were counted after females were transferred to new vials to determine the female fecundity. Unhatched eggs were counted the following day to calculate the proportion of oviposited eggs that hatched (i.e. hatching success). Vials were checked daily for eclosion, and egg-to-adult development time was recorded. Post-hatching viability was also calculated by dividing the number of eclosed adults by the number of hatched eggs. Females that did not lay any eggs were dissected, and their reproductive tracts were observed for the presence of sperm. Three females lacking stored sperm, indicating unsuccessful copulations, were excluded from analyses.

Statistical analysis of experiment 1

All data were analysed using R 2.12.2 (R Development Core Team, 2011). Male and female influence on early offspring viability was analysed using two-way ANOVAS for fecundity, hatching success, post-hatching viability

and development time. Two crosses failed to produce any adult offspring among all replicates. These missing cells were replaced by line-specific mean values of post-hatching viability or development time calculated using viability values from both male and female lines. For some oviposition vials, slightly more offspring were counted as adults than as eggs ($n = 7$ of 104 vials), resulting in proportional hatching success and post-hatching viability measurements in excess of one. Because the eggs in any vial had an equal chance of being undercounted, these data were analysed as is. Reported values are mean ± standard deviation, unless otherwise noted.

Experiment 2: reproductive success and offspring viability

Focal male flies originated from the outbred LHm population. Standard competitor males originated from a Ub-GFP isofemale line that was used in an effort to limit genetic variation among competitor males (e.g. Bjork *et al.*, 2007). Females originated from an inbred line of LHm created by three generations of full-sibling mating to reduce genetic variation in mate preference (e.g. Bjork *et al.*, 2007).

Outbred focal males ($n = 60$) were collected upon eclosion from LHm culture bottles. Inbred experimental females and standard competitor males were reared at a standard density of 50 larvae per 8-dram vial to minimize larval competition and phenotypic expression of inherent variance in condition. These rearing conditions significantly reduced phenotypic variation in thorax length of standard competitor males as compared to focal males (mean ± SD; competitors: 0.94 mm ± 0.03, focal: 0.89 mm ± 0.06; Fligner–Killeen nonparametric test for homogeneity of variance $\chi^2_1 = 33.40$, $P < 0.0001$). Eclosing flies were collected as described in experiment 1, and males were mated once prior to experimental crosses.

To assay contributions to variation in reproductive success among focal males in traits relevant to precopulatory sexual selection, post-copulatory sexual selection and viability selection, each focal male was experimentally mated to four different LHm females, with no more than one mating opportunity per day over six successive days. Subsequent to an initial mating to avoid virgin effects, focal males were subjected to test matings I, II and III, where the order of matings II and III was randomized among males in a fully balanced design:

(I) single mating – virgin female singly mated to focal male to assay (i) male attractiveness to a virgin female, (ii) single-mating productivity, (iii) hatching success, (iv) post-hatching viability and (v) development time

(II) focal male first – virgin female mated to focal male, then remated to standard competitor male to assay (i) male attractiveness to a virgin female, (ii) induced female refractoriness (i.e. mating latency with standard competitor male), (iii) first-male fertilization success, (iv) hatching success, (v) post-hatching viability and (vi) development time

(III) focal male second – virgin female mated to standard competitor, then remated to focal male to assay (i) male attractiveness to a nonvirgin female, (ii) second male fertilization success, (iii) hatching success, (iv) post-hatching viability and (v) development time

As an index of male 'attractiveness' (see Hosken *et al.*, 2008) to virgin females (test matings I and II), we quantified to the nearest minute the time elapsed from introduction of the focal male to the start of copulation (i.e. mating latency). Mating latency with nonvirgin females (III) and ability to induce refractoriness to remating in mates (II) was quantified as day of remating (females provided 6-h long opportunities to remate on each of three consecutive days following initial mating). Copulation duration was quantified as in experiment 1. As an index of body mass, the thorax length of each fly was measured after the completion of mating or oviposition.

Following the conclusion of their final mating, each female was transferred by aspiration to a new oviposition vial or plate once a day for 2 days to quantify female fecundity. Treatment I females oviposited in vials with standard cornmeal–molasses–agar medium supplemented with live yeast. Treatments II and III females oviposited on plates with apple juice–agar medium supplemented with live yeast because the autofluorescence of standard medium interferes with paternity assignment of eggs by fluorescent markers. After hatching was complete, first-instar larvae and the associated apple juice–agar medium were gently transferred from plates to vials with standard medium to allow for offspring development.

Offspring viability and fertilization success associated with each focal male were calculated by quantifying the number of offspring sired by the focal male at multiple stages of offspring development. For treatments II and III, eggs and eclosed adult offspring were counted under fluorescence to determine the paternity at egg and hatching stages; eggs fertilized by the focal male were unlabelled, and eggs fertilized by the competitor male fluoresced green. This enabled the quantification of paternity-specific offspring viability variables (i.e. hatching success, post-hatching viability and egg-to-adult development time) and the proportion of progeny sired by the focal male for each cross.

Statistical analyses of experiment 2

Univariate correlation or linear regression was conducted to determine the relationships between precopulatory, post-copulatory and offspring viability variables (Table 1). In addition to mating latency, precopulatory success included male size due to large-male advantages in gaining copulations (e.g. Partridge, 1988). Because paternity was determined at multiple offspring life stages,

Table 1 Mean and standard deviation of all study variables from first experiment, investigating the relationship between reproductive success and offspring quality. Number of males successfully completing each treatment reported in column headers; deviations from these sample sizes noted as applicable. Note that cases of paternity = 1 for treatments II and III have been omitted.

	Mating Treatment		
	I – single mating n = 55 males	II – P_1 n = 46 males	III – P_2 n = 24 males
Precopulatory variables			
Focal male thorax length (mm)	0.89 ± 0.06 (n = 50)	0.89 ± 0.06 (n = 42)	0.88 ± 0.06
Mating latency	18.69 min ± 34.11	16.52 min ± 32.00	1.08 days ± 0.28
Post-copulatory variables			
F_1 or F_2	–	0.62 ± 0.15	0.92 ± 0.10
P_1 or P_2	–	0.44 ± 0.17	0.87 ± 0.15 (n = 23)
Refractoriness (days)	–	1.41 ± 0.50	–
Offspring viability variables			
Average development time (days)	12.56 ± 0.37	11.58 ± 0.38	11.38 ± 0.35
Female fecundity	36.16 ± 16.73	99.39 ± 24.95	93.25 ± 22.33
Hatching success	0.86 ± 0.13	0.74 ± 0.14	0.85 ± 0.11
Post-hatching viability	1.04 ± 0.17	0.71 ± 0.24	0.84 ± 0.15

references to proportion of progeny sired by focal males follow García-González (2008): paternity calculated from eggs is referred to as F_1 and F_2, indicating fertilization success, whereas paternity as determined in adult offspring is designated with the traditional P_1 and P_2. Analyses involving paternity were separately conducted using both methods of calculating paternity. Multivariate tests of relationships (e.g. canonical correlation) had prohibitively low power because focal males missing one or more variables would require exclusion (e.g. focal male mated in all treatments, but was lost prior to body size measurement). Complete data sets across the three treatments were gathered for only 12 males. As such, relationships between pre- and post-copulatory episodes, or sexually selected (i.e. pertaining to both precopulatory and post-copulatory variables) and viability selected (i.e. pertaining to offspring viability variables) episodes, were assessed with stepwise elimination of nonsignificant variables in linear models. Model fitting and simplification followed Crawley (2007). Full models consist of each post-copulatory variable predicted by both precopulatory variables, or each viability variable predicted by all sexually selected variables. In a few cases, residuals of minimal linear models remained heteroscedastic despite variable transformations. Because all such minimal models retained only one predictor variable, significance was tested with Spearman's rank correlations. One outlier for treatment I hatching success and one outlier for treatment II F_2 altered the retention of minimal model terms; the following results are presented without these values. Variables in univariate correlations were transformed for normality or analysed using Spearman's rank correlation. Cross-episode correlations and regressions were tested for significance with alpha values corrected for the false discovery rate (Benjamini & Hochberg, 1995) using 15 tests for fertilization success or paternity success.

The influence of sexually and viability selected traits on male fitness was assessed using general linear models, where each mating treatment (i.e. I, II or III) was analysed individually. Rate-sensitive fitness scores were calculated by dividing the number of each male's eclosed offspring by his offspring's average development time. Our fitness metric included a rate component because larvae develop in a substrate that diminishes in quality over time, suggesting that faster development time may be beneficial (Roff, 2002). Separate models were fit using our rate-sensitive fitness metric, as well as the number of offspring or development time as single response variables. Predictor variables were the same as in the cross-episode analyses. Full models included all main effects and any significant interactions from preliminary models as explanatory variables. Copulation duration and female body size were also included as covariates. Although results reflect models fit with untransformed paternity values, arcsine-square-root transformation of paternity scores yielded minimal models retaining the same main effects. Relative contribution of minimal model terms to overall variance in male fitness was calculated by partitioning variance based on partial correlation coefficients following Legendre & Legendre (2000).

Repeatability (Lessells & Boag, 1987) of offspring viability variables across the three matings was calculated to further explore male influence on offspring viability. Specifically, repeatability of fitness, fecundity, hatching success and post-hatching viability were calculated using ANOVA with mating treatment (i.e. I, II, III) nested within male identity. Male *D. melanogaster* have been shown to differentially transfer sperm (Lüpold *et al.*, 2011) and amounts of seminal peptides that induce egg production (Sirot *et al.*, 2011) to virgin vs. previously mated females. Because differential ejaculate investment may influence fitness and fecundity in our virgin (i.e. treatments I

and II) vs. nonvirgin (i.e. III) mating treatments, repeatability was calculated with standardized response variables (i.e. mean = 0, SD = 1) for these two variables.

Because we could not distinguish whether observations where focal males achieved 100% paternity were due to complete sperm competitive success of the focal male or failure of standard competitors to transfer sperm (focal male $F_1 = 1$ for 1 of 47 females in treatment II; focal male $F_2 = 1$ for 12 of 36 females in treatment III), analyses were completed with and without these data. As in experiment 1, slightly more offspring were counted as adults than as eggs in some oviposition vials ($n = 13$ of 165 vials) and these data were analysed as is.

Results

Experiment 1: male and female influence on offspring viability

Significant male × female interactions explained variance in both fecundity and hatching success, as well as a female line main effect on fecundity and a male line main effect on hatching success (Table 2).

Experiment 2: reproductive success and offspring viability

Impact of traits on male fitness

In minimal linear models for rate-dependent fitness (Table 3) and offspring number (Table S1), variance in response variables was significantly influenced by female fecundity, hatching success, post-hatching viability and fertilization success for the competitive matings. Main effects unique to individual models and interaction terms are discussed in more detail below.

For the single-mating treatment (I), a few interactions significantly influenced variance in male fitness (Table 3) and total number of eclosed offspring (Table S1). Two-way interactions between fecundity and hatching success or post-hatching viability reflect their synergistic influence on numbers of eclosed offspring. At higher fecundity levels, the same proportional increase in hatching success or post-hatching viability leads to a larger increase in the absolute number of eclosed offspring and in fitness. In the fitness model, the hatching success × post-hatching viability interaction is due to increases in post-hatching viability positively influencing fitness at low levels, but negatively impacting it at high hatching success (Fig. S1c). This may be explained by larval competition reducing fitness where more offspring successfully hatch. Indeed, a trade-off exists between the number of hatched offspring and survival for this treatment ($\rho_{49} = -0.31$, $P = 0.03$). No explanatory variables remained in the minimum adequate model for variance in development time.

In treatment II, variance in male fitness (Table 3) and total number of focal male's eclosing progeny (Table S1) were explained by female size in addition to the main effects common to all treatments. A number of synergistic interactions were also present in minimal models explaining variance in fitness (i.e. F_1 × post-hatching viability) and offspring number (i.e. hatching success × post-hatching viability, F_1 × post-hatching viability, and F_1 × hatch). A female size × F_1 interaction arose in both treatment II models and appeared to also be due to a synergistic relationship, with increases in F_1 benefiting males more when they were mated to a larger female, although this pattern was less clear for offspring number (Fig. S1a,b). The minimal model explaining variance in development time was not significant ($R^2 = 0.22$, $F_{4,33} = 2.384$, $P = 0.07$).

In addition to the main effects shared by all models for male fitness (Table 3) and number of eclosed offspring (Table S1), variance in male fitness in treatment III was also significantly influenced by copulation duration and male size. Variance in offspring number was explained by the common model terms as well as by synergistic interactions of female fecundity with hatching success and post-hatching viability. The minimal model on development time of a focal male's progeny included copulation duration, male size and female size (Table S1). A single interaction, male size × copulation duration, was present in models for both male fitness and development time; the mechanism underlying these interactions is unclear (Fig. S1d,e).

Including cases of 100% paternity resulted in retention of many of the same main effects in most minimal models (Table S2). In a few cases, new main effects were included (treatment II number of offspring: female refractoriness; treatment III number of offspring: male size and copulation duration) or minimal models were nonsignificant (treatment III development time).

Relationships between episodes of selection

No significant relationships were found between precopulatory and post-copulatory sexual selection after

Table 2 Results of Model II analysis of variance, analysing female fecundity and hatching success[3] (transformed to approach normality) among isoline crosses. Type III sums of squares were used to account for unbalanced data.

Source of variance	SS	d.f.	*F*	*P*
a) Female fecundity				
Female line	12 704	5	4.09	0.01
Male line	5471	5	1.76	0.16
Female line × male line	15 526	25	2.12	0.01
Error	24 864	85		
b) Hatching success				
Female line	0.87	5	1.94	0.12
Male line	1.22	5	2.73	0.04
Female line × male line	2.23	25	1.80	0.03
Error	3.37	68		

Table 3 Minimal general linear models of effect of sexual and viability selected variables on focal male fitness (i.e. number of offspring eclosed/average development time) and partitioned variance among minimal model terms for competitive matings. All observations with 100% paternity have been excluded. Note: *Variation common to multiple terms* quantifies the overlap of variance in the response variable explained by multiple predictor variables (Legendre & Legendre, 2000), which is not equivalent to unexplained variance (i.e. residual variance).

Source of variance	Estimate	SE	*t*	*P*	Per cent variance explained (%)
a) Treatment I - single mating					
Focal male fitness (n = 50 males, minimal model R^2 = 0.996, $F_{6,43}$ = 1669, P < 0.0001)					
Hatching success	−2.32	0.63	−3.68	< 0.0001	0.14
Post-hatching viability	−2.24	0.36	−6.31	< 0.0001	0.43
Female fecundity	−0.07	0.01	−4.93	< 0.0001	0.26
Post-hatching viability × female fecundity	0.08	0.01	11.54	< 0.0001	1.46
Post-hatching viability × hatching success	2.28	0.36	6.33	< 0.0001	0.43
Female fecundity × hatching success	0.07	0.01	5.85	< 0.0001	0.37
Variation common to multiple terms					96.42
Residual variance					0.49
b) Treatment II – competitive mating, focal male mates first					
Focal male fitness (n = 38 males, minimal model R^2 = 0.977, $F_{7,30}$ = 182.2, P < 0.0001)					
Proportion progeny (F_1)	−30.10	10.75	−2.80	0.01	0.62
Hatching success	3.77	0.25	15.13	< 0.0001	20.83
Post-hatching viability	−2.51	0.53	−4.70	< 0.0001	1.93
Female fecundity	0.03	0.002	15.11	< 0.0001	20.78
Female size	−14.74	5.96	−2.35	0.03	0.41
F_1 × female size	25.49	10.18	2.42	0.02	0.44
F_1 × post-hatching viability	10.33	1.02	10.09	< 0.0001	9.21
Variation common to multiple terms					42.93
Residual variance					2.83
c) Treatment III – competitive mating, focal male mates second					
Focal male fitness (n = 21 males*, minimal model R^2 = 0.991, $F_{7,13}$ = 206.4, P < 0.0001)					
Focal male copulation duration	0.76	0.13	6.06	< 0.0001	4.47
Proportion progeny (F_2)	5.13	0.58	8.77	< 0.0001	7.41
Hatching success	5.74	0.44	12.94	< 0.0001	17.48
Post-hatching viability	5.71	0.35	16.28	< 0.0001	25.20
Female fecundity	0.06	0.003	17.96	< 0.0001	35.96
Focal male size	23.24	3.67	6.27	< 0.0001	4.63
Focal male size × focal male copulation duration	−0.91	0.15	−6.08	< 0.0001	4.59
Variation common to multiple terms					−3.11
Residual variance					3.37

*One high leverage point removed, its inclusion retains post-hatching viability × female fecundity.

exclusion of one male with low F_2 (0.56, all other values > 0.78). Spearman's rank correlations between variables associated with post-copulatory selection were nonsignificant (F_1 and F_2: ρ_{16} = 0.23, P = 0.38; F_1 and refractoriness: ρ_{45} = −0.27, P = 0.07). A positive relationship was found between F_1 and hatching success after the removal of a single observation with unusually low hatching success (r = 0.34, t_{39} = 2.24, P = 0.03). Inclusion of cases with 100% paternity yielded qualitatively similar results, with the exception of the relationship between F_2 and male size becoming statistically significant (r = 0.42, t_{33} = 2.63, P = 0.01). Although 95% confidence intervals around the linear model coefficient estimates (i.e. effect size) for both relationships did not include 0 (F_1 and hatching success: coefficient estimate = 0.28, 95% CI = 0.07–0.49; F_2 and male size: coefficient estimate = 0.42, 95% CI = 0.15–0.70), neither relationship remained significant after FDR correction.

It is possible that our inability to distinguish among eggs fertilized by the focal male and unfertilized eggs could influence the above relationships. If unfertilized eggs had been scored as focal male progeny, fertilization success (i.e. F_1) would be artificially inflated, but offspring hatching success would decrease, weakening the strength of the correlation. A comparison of hatching success of focal males and standard competitors across treatments suggests that this may be the case (focal hatching success = 0.79 ± 0.13, competitor hatching success = 0.98 ± 0.20, one-sided Wilcoxon rank sum test,

$W_{71} = 484$, $P < 0.0001$). As such, the results discussed in this section are conservative estimates of these cross-episode relationships.

Notably, different results were obtained when relationships between precopulatory and post-copulatory variables were evaluated using P_1 or P_2 as opposed to F_1 or F_2. Relationships became stronger between first-male paternity and hatching success ($r = 0.40$, $t_{38} = 2.72$, $P = 0.01$; outlier and two high leverage points excluded) and appeared between paternity and post-hatching viability (P_1: $r = 0.54$, $t_{38} = 4.08$, $P = 0.0002$; P_2: $\rho_{21} = 0.56$, $P = 0.005$), as well as between second male paternity and male size ($r = 0.48$, $t_{21} = 2.51$, $P = 0.02$). Paternity relationships remain significant after FDR correction, except for that between P_2 and male size.

Repeatability of offspring viability across matings

Repeatability of fitness, fecundity, hatching success or post-hatching viability across the three test matings in the experiment 2 was not significant (all repeatabilities <0.11, $F < 1.35$, $P > 0.23$).

Discussion

We found that variance in male fitness was significantly influenced by both sexual (i.e. fertilization success) and offspring viability selection (i.e. hatching success and post-hatching viability), the latter was particularly influential when focal males were first to mate against a standard competitor (Table 3; experiment 2, treatment II). Interestingly, viability selection may explain the differences we found in presence and strength of cross-episode relationships when 'fertilization success' was measured at different points of offspring development. In three cases, relationships became statistically significant after FDR correction when paternity was measured in adult offspring (i.e. with P_1 or P_2) rather than eggs (i.e. with F_1 or F_2), results we interpret as arising from differential offspring viability influencing P_1 and P_2.

This result reinforces previously identified concerns about the interpretation of cross-episode relationships with paternity (Gilchrist & Partridge, 1997; García-González, 2008). Where studies do not control for offspring viability differences methodologically (e.g. as controlled in Danielsson, 2001; Evans *et al.*, 2003; Fisher *et al.*, 2006; Bilde *et al.*, 2009) or statistically (e.g. as controlled in Hosken *et al.*, 2003; Pischedda & Chippindale, 2006), correlations with paternity may at least partially represent differential offspring viability. Where relationships have been found between P_2 and male attractiveness (Lewis & Austad, 1994; Hosken *et al.*, 2008; Bretman *et al.*, 2009; Fricke *et al.*, 2010), presence of significant differential viability would reduce support for a 'good sperm' interpretation (e.g. as suggested by Lewis & Austad, 1994; Hosken *et al.*, 2008) in favour of a model that incorporates offspring viability (e.g. genetic compatibility or good genes).

The lack of repeatability of offspring viability across a focal male's matings in experiment 2 further suggests that caution is warranted when applying correction factors from a separate, single mating to P_1 or P_2 values to account for differential offspring viability. To illustrate this point, when treatment I egg-to-adult viability was used to correct treatment II and III paternity values (i.e. paternity calculated from adult offspring and multiplied by treatment I viability), the same statistically significant relationships were found as when investigating relationships with raw paternity scores (results not shown). This suggests that when viability of offspring is not repeatable across matings, the application of viability correction factors may not adequately control for the influence of differential offspring viability on paternity measurements. Previous studies have weighted paternity on the population (e.g. Chang, 2004), or genetic isoline level (e.g. Clark *et al.*, 1999; Fricke *et al.*, 2010), as opposed to the individual male level, as in the present study. Because repeatability of viability within lines is rarely reported, it is difficult to determine whether applying correction factors at this level more adequately controls for confounding effects.

Two cross-episodic trends were found when using fertilization success, which did not remain significant after correction for the false discovery rate. Positive relationships between fertilization success and either hatching success (treatment II) or male size (treatment III) would be consistent with two models. First, under the 'good sperm' model of polyandry, fertilization success is influenced by overall male condition (Sivinski, 1984; Madsen *et al.*, 1992; Yasui, 1997), where high-condition males (e.g. larger) achieve increased post-copulatory success and also produce more robust offspring (e.g. with improved embryo viability). Second, cryptic female choice theory posits that females bias fertilizations in favour of preferred males, which includes more attractive or genetically compatible mates (Eberhard, 1996).

In order to avoid complex male × male × female interactions inherent to competitive matings (e.g. in sperm competitive success; Bjork *et al.*, 2007), it was necessary to limit genotypic and phenotypic variation in females and standard competitor males in our reproductive success experiment. It is possible that the limited number of significant relationships we found, particularly between sexually selected episodes, was due to reduced phenotypic variance through inbreeding of standard competitors and females. We consider this unlikely for most of the examined traits due to the presence of appreciable variation in most variables (Table 1, but see limited variation in development time and treatment III mating latency). Moreover, although some studies using outbred *Drosophila* have found relationships between pre- and post-copulatory success (e.g. between P_2 and mating latency: Hosken *et al.*, 2008; Bretman *et al.*, 2009; Fricke *et al.*, 2010), others have found no correlation between these episodes (Pischedda & Rice, 2012).

Our finding that differential offspring viability significantly explains variance in male fitness reinforces previous cautions about interpreting relationships with paternity as measured in adult offspring (Gilchrist & Partridge, 1997; García-González, 2008). Moreover, the lack of repeatability in offspring viability among focal males' matings and a significant male × female effect on hatching success and post-hatching viability illustrate the complexity of quantifying reproductive success, as well as identifying relationships between episodes of selection.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Non-synergistic interactions significant in minimal linear models.

Table S1 Minimal general linear models of effect of sexually and viability selected variables on number of offspring sired by the focal male as well as offspring development time.

Table S2 Minimal general linear models of effect of sexually and viability selected variables on focal male fitness, including cases of 100% paternity.

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