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Additive genetic variance in polyandry enables its evolution, but polyandry is unlikely to evolve through sexy or good sperm processes

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Abstract

Polyandry is widespread despite its costs. The sexually selected sperm hypotheses ('sexy' and 'good' sperm) posit that sperm competition plays a role in the evolution of polyandry. Two poorly studied assumptions of these hypotheses are the presence of additive genetic variance in polyandry and sperm competitiveness. Using a quantitative genetic breeding design in a natural population of *Drosophila melanogaster*, we first established the potential for polyandry to respond to selection. We then investigated whether polyandry can evolve through sexually selected sperm processes. We measured lifetime polyandry and offensive sperm competitiveness (P_2) while controlling for sampling variance due to male × male × female interactions. We also measured additive genetic variance in egg-to-adult viability and controlled for its effect on P_2 estimates. Female lifetime polyandry showed significant and substantial additive genetic variance and evolvability. In contrast, we found little genetic variance or evolvability in P_2 or egg-to-adult viability. Additive genetic variance in polyandry highlights its potential to respond to selection. However, the low levels of genetic variance in sperm competitiveness suggest that the evolution of polyandry may not be driven by sexy sperm or good sperm processes.

Introduction

Polyandry, when females mate with more than one male within a single reproductive episode, frequently leads to the overlap of ejaculates within the female reproductive tract, hence allowing selection to continue after mating through sperm competition and cryptic female choice (Parker, 1970; Eberhard, 1985). Sperm competition promotes male traits that alter sperm utilization, fertilization and female remating by influencing female physiology and behaviour (Parker, 1970; Simmons, 2001). Polyandry can also generate sexual conflict over mating decisions, which may lead to antagonistic coevolution of male manipulation and female resistance when the two sexes have different optimal mating rates (Arnqvist & Rowe, 2005). Thus, polyandry has profound ecological and evolutionary consequences (Kvarnemo & Simmons, 2013; Parker & Birkhead, 2013).

Polyandry results in increased time and energy expenditure required to obtain mates, increased risk of exposure to predators and/or sexually transmitted diseases (e.g. Rowe *et al.*, 1994; Hurst *et al.*, 1995; Watson *et al.*, 1998; Knell & Mary Webberley, 2004), and may entail further direct costs arising from sexual conflict, for instance in the form of decreased lifespan (Chapman *et al.*, 1995). Increased sexual activity in females can also have negative trans-generational effects on fitness (Dowling *et al.*, 2014). Despite these costs, theoretical and empirical findings suggest that polyandrous females may acquire direct benefits (Arnqvist & Nilsson, 2000), indirect (genetic) benefits (Newcomer *et al.*, 1999; Jennions & Petrie, 2000; Garcia-Gonzalez & Simmons, 2005; Simmons, 2005; Slatyer *et al.*, 2012;

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Klemme *et al.*, 2014) and/or beneficial indirect genetic paternal effects (Garcia-Gonzalez & Simmons, 2007a). Such benefits may offset the costs of polyandry. Females are thus expected to adopt an optimal mating frequency whereby they balance the costs and benefits associated with mating (Arnqvist & Nilsson, 2000).

Given the costs associated with multiple mating, a series of explanations have been put forward to explain the evolution of polyandry when females obtain no direct benefits. One explanation that has received much attention focuses on the acquisition of sexy or good genes for offspring (Yasui, 1998). Analogous to the pre-copulatory sexy sons model of female choice, the sexy sperm hypothesis suggests that by facilitating sperm competition, polyandrous females will have a selective advantage over monandrous females as their sons will be sired by competitively superior males and inherit genes for enhanced sperm competitiveness. Polyandrous females will also produce daughters that carry genes for increased mating frequency, generating the coevolution of sperm competitiveness and polyandry through Fisherian 'runaway' selection (Keller & Reeve, 1995; Evans & Simmons, 2008; Bocedi & Reid, 2014; Reid *et al.*, 2014). The good sperm hypothesis posits that females can accrue genetic quality for their offspring if males of high overall genetic quality are superior sperm competitors because the offspring sired by these successful males will exhibit high viability (Yasui, 1997). Importantly, indirect selection for polyandry via the sexy and good sperm processes is predicted only when there is additive genetic variance in both female mating frequency and sperm competitiveness and when these traits exhibit genetic covariance (Evans & Simmons, 2008). Despite great theoretical interest (Curtsinger, 1991; Keller & Reeve, 1995; Yasui, 1997; Evans & Simmons, 2008; Bocedi & Reid, 2014), empirical demonstrations of the key assumptions of the sexually selected sperm models are scarce and generally inconclusive.

Both the sexy sperm and good sperm models assume heritability in polyandry; in order to respond to sexual selection, there must be additive genetic variation in female mating frequency. However, findings so far on the genetic architecture of polyandry are inconclusive (for a review, see Evans & Simmons, 2008). Whereas some studies suggest a genetic basis for polyandry (Torres-Vila *et al.*, 2002; Linder & Rice, 2005; Evans & Simmons, 2008), others do not (McFarlane *et al.*, 2011; Evans & Gasparini, 2012). The conflicting findings on the subject thus far make it difficult to draw firm conclusions on the potential for selection to act on female mating rates.

Another key assumption of the sexually selected sperm models – additive genetic variance for sperm competitiveness – is mixed (Hughes, 1997; Simmons, 2003; Dowling *et al.*, 2010; but see Radwan, 1998; and Friberg *et al.*, 2005). Studies of individual sperm traits that contribute to sperm competitiveness report evidence for additive genetic variance (Arnqvist & Danielsson, 1999; Hunter & Birkhead, 2002; Miller & Pitnick, 2002; Simmons & Kotiaho, 2002; Preston *et al.*, 2003; Garcia-Gonzalez & Simmons, 2007b; Simmons & Garcia-Gonzalez, 2008). There are also some studies that have manipulated the strength of post-copulatory sexual selection, finding responses in sperm competitiveness (Hosken *et al.*, 2001; Simmons & Garcia-Gonzalez, 2008; Firman & Simmons, 2011). However, these responses are often decreases in sperm competitiveness under relaxed selection rather than increased competitiveness with intensified sperm competition (Nandy *et al.*, 2013; Pitnick *et al.*, 2001b; but see Konior *et al.*, 2005). Several studies on male reproductive genes have also mapped polymorphisms that contribute to male fertilization success independent of the competitor male genotype (Zhang *et al.*, 2013) and female genotype (Reinhart *et al.*, 2015). Nonetheless, studies investigating the genetic basis of sperm competitiveness *via fertilization success* have both provided support for additive genetic variance in sperm competitive ability (Hosken *et al.*, 2001; Simmons & Garcia-Gonzalez, 2008; Firman & Simmons, 2011) and failed to detect a genetic basis for sperm competitive ability (Arbuthnott *et al.*, 2014; see also review by Simmons & Moore, 2009).

Accurate measurement of sperm competitiveness also requires direct estimates of fertilization success at conception. For practical reasons, studies using species where fertilization occurs internally often rely on the proxy of progeny produced at birth as a measure of fertilization success. In addition to variation in a male's ability to gain fertilizations when in competition, the number of adult progeny produced by a male will be affected by egg-to-adult viability of his offspring. Without controlling for variation in pre-adult viability, estimates of fertilization success may be influenced by heritable differences in pre-adult viability, rather than by heritable differences in sperm competitiveness (Gilchrist & Partridge, 1997; Garcia-Gonzalez, 2008a). The effect of intrinsic sire effects on egg-to-adult viability must therefore be accounted for in experiments that aim to assess additive genetic variance in sperm competitiveness.

In this study, we used a quantitative genetic approach to first examine the evolutionary potential of polyandry (the potential for female mating frequency to respond to selection) and second to test the key assumptions of the sexy sperm and good sperm processes in a natural population of *Drosophila melanogaster*. In this species, females mate multiply and are known to suffer a mating-related reduction in lifespan due to the toxic effects of seminal fluid proteins transferred from males to females during mating (Chapman *et al.*, 1995; Pitnick & Garcia–Gonzalez, 2002; Wigby & Chapman, 2005). There is a lack of evidence for any benefits that might outweigh these costs of mating (e.g. Brown

et al., 2004; Byrne & Rice, 2005; Orteiza *et al.*, 2005). Further work is therefore required to both establish the evolutionary potential of female mating frequency and the processes that could at least partly favour the maintenance of polyandry, even if the benefits cannot fully compensate the costs.

Using a full-sib/half-sib breeding design, we calculated additive genetic variance in female lifetime mating frequency. We then calculated additive genetic variation in sperm competitiveness via P_2, to measure the genetic covariance between polyandry and sperm competitiveness that is predicted by the sexy sperm process. We also controlled for confounding effects of nonsperm representation on distributions of fertilization success by ensuring that only females where both the first and second male achieved sperm representation were included in the analysis. Finally, we measured intrinsic sire effects on egg-to-adult survival in second males to control for the confounding effect of differential egg-to-adult viability on P_2 estimates. We estimated additive genetic variance in intrinsic male effects on egg-to-adult viability and tested whether good sperm competitors generate offspring of superior genetic quality as predicted by the good sperm model.

Materials and methods

Breeding design

Flies for the parent generation were taken from a large (~500 flies) laboratory population of sixth-generation descendants of wild-type (*wt*) *D. melanogaster* collected near Innisfail in Northern Queensland, Australia. To produce parents of focal females, grape agar plates were placed in the population cage for 4 h. The following day, we collected first-instar larvae and transferred them to vials at a standard density of 50 larvae per vial. Vials contained 10 mL of sugar–maize medium. Offspring were collected 9–11 days later under CO_2 anaesthesia within 8 h of eclosion and transferred to single sex vials. Males were kept at a density of 10 per vial and females at 5 per vial. Parental generation matings were carried out when flies were 3–4 days old.

Each male was mated to three virgin females to generate families of paternal half-sibs and maternal full-sibs. After mating, females were transferred to individual vials and moved to new vials every 48 h for 4 days. During peak eclosion, eight virgin female offspring (daughters) from each full-sibling dam family were randomly collected; four were included in the lifetime mating frequency assay and four were frozen and later used to estimate full-sibling dam family average female body size. Four virgin males (sons) were also collected at random from each full-sibling dam family for use in the P_2 and egg-to-adult viability assays. Mating opportunities for female lifetime mating frequency estimates began when females were 3–5 days old. Thus, variability in the age of individuals did not contribute to the variation in female mating frequency (Fricke *et al.*, 2013). P_2 matings were also carried out when males were 3–5 days old. Egg-to-adult viability scores for the focal P_2 males were obtained 72 h after the P_2 mating. Egg, larvae and adult flies were maintained at 25 °C on a 12:12-h light/dark diurnal cycle throughout all experiments.

Lifetime mating frequency of *wt* females

Lifetime mating frequency was recorded for 775 daughters distributed among 72 sire families and 198 dam families over 9 months. We kept females in individual vials and transferred them to fresh food vials once a week for the duration of their lifetime. Each female was given the opportunity to mate with a previously unmated male for 1 h every Monday, Wednesday and Friday over her entire lifespan. Successful copulations were recorded and all pairs were separated after 1 h. In order to minimize sampling variance arising from male effects (e.g. receptivity-inhibiting seminal fluids) on female remating rates (Garcia-Gonzalez & Evans, 2011; Travers *et al.*, 2015), virgin males were taken from one of 10 isogenic lines for each mating. Each isogenic line was generated through a protocol of full-sibling matings started with one founder pair of flies taken from a replica of an LH_M population (see Byrne & Rice, 2005 for details). The isogenic lines were obtained through a protocol of brother–sister matings for 16 generations, followed by several generations of within-line matings (approximately 15 individuals from each vial for each new generation), before reinstating a full-sibling mating protocol for another 21 generations. Before the beginning of the experiment, isogenic lines were mass-bred into population cages to allow the collection of sufficient numbers of flies needed for each mating trial. We standardized the identity of the male partner by randomly selecting males from the same isogenic line in each mating opportunity (e.g. isogenic line 1 in the first opportunity for all females, isogenic lines 2, 3, 4, etc., for the 2nd, 3rd, 4th, etc., mating opportunity). To obtain males for each mating opportunity, larvae were collected from population cages on grape agar plates and sexually naïve males collected at peak eclosion. We used 2- to 3-day-old males for the mating trials.

Sperm competitiveness (P_2) of *wt* males

P_2 was assessed for 242 sons distributed over 53 sire families and 126 dam families. We estimated the additive genetic variance of the sperm competition phenotype measuring P_2 values after conducting double mating trials using standardized tester individuals (Fricke *et al.*, 2010). We used females and rival males from isogenic lines to reduce sampling variance associated with the use of random tester individuals

(Garcia-Gonzalez, 2008b; Garcia-Gonzalez & Evans, 2011). Females were generated from isogenic lines with recessive red eyes, and rival males were taken from an isogenic line with a dominant brown eye colour mutation (different isogenic lines were used to source rival males and females).

For the rival (first) matings, mass matings were conducted in bottles provisioned with food for groups of 80 isogenic males combined with 40 isogenic females per bottle. Males and females were lightly aspirated by CO_2 from their single sex-holding vials and combined at a 2 : 1 ratio for 1.5 h. This time period was selected because previous studies have shown that most females mate within 2 h under these conditions, and very few mate more than once (Prout & Bundgaard, 1977; Hughes, 1995; Holland & Rice, 1999; Dowling *et al.*, 2014). We chose the more conservative time period of 1.5 h rather than 2 h to further minimize any chance for double matings. After mating, males were discarded and females were placed into individual food vials for 48 h.

Methods used in sperm competition trials have been criticized for their lack of control for nonsperm representation (Garcia-Gonzalez, 2004). For example, insemination failure or male sterility in experimental estimates of sperm competitiveness may contribute to the probability of obtaining distributions of P_2 that do not accurately characterize the underlying patterns of sperm use (Garcia-Gonzalez, 2004). Therefore, failure to detect occurrences of infertile matings in sperm competition trials may introduce inaccuracy into estimates of additive genetic variance in sperm competitiveness. To avoid this problem, before the beginning of the P_2 assay, each isogenic female vial was inspected for the presence of larvae to ensure that the female was successfully inseminated during the first mating session. Females from vials with no larval presence were not included in the P_2 assay.

Forty-eight hours after the P_1 mating, each isogenic female was placed in a vial with a red eye recessive *wt* focal male. Pairs were observed continuously for 3 h and then scan-sampled every 10 min for another 7 h. After a mating pair disengaged, the female was immediately removed from the vial and placed into a fresh vial. Females were then transferred to new food vials every 24 h for the first 3 days and then once every 48 h until 14 days after the P_2 mating. After all flies had emerged, progeny were scored for eye colour. The proportion of red-eyed progeny was taken as a measure of P_2. Given the strong last male sperm precedence in *D. melanogaster* (e.g. Gromko *et al.*, 1984; Hughes, 1997), females that produced only brown-eyed progeny were highly likely to have experienced insemination failure in the second mating and were therefore removed from the analysis. The focal males were kept in individual vials for later use in the egg-to-adult viability assay.

Egg-to-adult viability of *wt* males

Twenty-four hours after mating in the P_2 assay, each focal male was mated monogamously to a previously unmated isogenic female from the same isogenic line as the females used in the P_2 assay. Males and females were paired in individual yeasted vials for 48 h before the assay commenced to ensure that the pair was mated and the female ready to oviposit. To measure egg-to-adult viability of offspring from the focal males, each pair was transferred to a vial with blue food dye for 24 h to allow the female to oviposit. The eggs laid in this period were counted and the number of adults that emerged after 14 days was used to calculate egg-to-adult viability.

Statistical analyses

The genetic basis of polyandry was determined by conducting independent analyses on two measures of female lifetime mating frequency: absolute mating frequency (the total number of lifetime matings) and the proportion of lifetime matings (total matings/mating opportunities). For both analyses, a subset of data ($n = 625$) was generated from the full data set to include only females that had at least two mating opportunities and mated at least once. This subset also eliminated females that escaped or were accidentally killed (17% of all females). Thus, only females that had lived a 'natural' lifespan were included in the final data set. For P_2 and egg-to-adult viability analyses, males from instances where no offspring had been produced in the egg-to-adult viability assay (viability = 0) were removed from the analysis as these instances cannot be distinguished from failed inseminations (9% of all males). Our final sample size for P_2 and egg-to-adult viability was thus 205 males distributed across 53 sire families.

The lme4 package (Bates *et al.*, 2014) implemented in R 3.03.3 (R Core Team, 2015) was used to fit standard nested mixed models for a paternal half-sibling design. For the total number of lifetime matings, hypothesis testing was carried out using a linear mixed model (LMM) on the log-transformed number of lifetime matings was fitted using the lmer function with sire and dam nested within sire as random effects and longevity and body size as fixed effects. We also included the date of first mating opportunity to control for temporal variation in female mating frequency throughout the experiment. Significance of fixed effects was tested using Wald chi-square tests implemented in the ANOVA function of the car package (Fox & Weisberg, 2011). Body size had no significant effect and was removed from the model. Significance of the sire and dam variance components was determined using likelihood-ratio tests.

Hypothesis testing was carried out on transformed data (e.g. using a binomial distribution family for P_2

which in its untransformed state is a proportion), whereas genetic parameters were calculated from untransformed data using REML, as explained below. Sire and dam effects for all traits measured as proportions (female lifetime mating proportion, P_2 and egg-to-adult viability) were estimated using binomial generalized linear mixed models (GLMM) using the glmer function with sire and dam nested within sire as random effects. Longevity and body size were included as fixed effects in the female lifetime mating proportion model. All females were given the first opportunity to mate when they were 3–5 days old, but for logistical reasons, there was variation among sire and dam families in the date we started the assays. To control for potential temporal variation, we included assay start date as a fixed effect. Body size had no significant effect and was not included in the final model. Significance of the sire and dam nested within sire variance components for female mating proportion was determined using likelihood-ratio tests. To account for overdispersion in models for P_2 and egg-to-adult viability, we included an observation-level random effect (Browne *et al.*, 2005) and determined the significance of variance components for P_2 and egg-to-adult viability using randomization tests with 1000 iterations on each variance component. Egg-to-adult viability was included as a fixed effect in the P_2 analysis to account for heritable differences in egg-to-adult survival that can influence paternity estimates (Gilchrist & Partridge, 1997; Garcia-Gonzalez, 2008a). All P_2 matings took place when focal males were 3–5 days old, but again there was a variation among sire and dam families in the date the assays were run. Thus, we included date as a fixed effect in P_2 and egg-to-adult viability models to control for temporal variation.

Genetic parameters for all traits were calculated using restricted maximum likelihood (REML) from LMMs on untransformed data using the lmer function. We calculated genetic parameters from untransformed data because many of these parameters are meaningless for comparative purposes if variance components are extracted when data are transformed (Hansen *et al.*, 2011; Houle *et al.*, 2011; Garcia-Gonzalez *et al.*, 2012). Observational variance components were estimated from minimal models including only significant fixed effects. We also calculated variance estimates for P_2 on the logit scale using an LMM on logit-transformed P_2 values as recommended by Engqvist (2013). Narrow-sense heritabilities (h^2) of female lifetime number of matings and mating proportion, P_2 and egg-to-adult viability were estimated from the ratio of additive genetic variance (V_A: four times the sire variance component) to total phenotypic variance. Mean-standardized measures of evolvability were calculated, namely the coefficient of additive genetic variation ($CV_A = (\sqrt{V_A})/\overline{X}$, where $\overline{X}$ is the phenotypic mean of the trait mean) and I_A ($V_A/\overline{X}^2$), an estimate of the expected proportional change under a unit strength of selection (Houle, 1992; Hansen *et al.*, 2011; Garcia-Gonzalez *et al.*, 2012). CV_p and CV_r were also calculated as described in Garcia-Gonzalez *et al.* (2012). Standard errors for all quantitative genetic parameters were calculated by jackknifing across sire families (Roff, 2006).

We examined whether polyandry can evolve through the sexy sperm process with a Pearson correlation on sire family means of residual P_2 (P_2 values corrected for significant covariates) and residual female lifetime mating frequency (lifetime mating proportion corrected for longevity).

Results

We found considerable phenotypic variation in female lifetime number of matings (mean ± SD = 3.59 ± 1.6; range = 1–9). Not surprisingly, there was a significant effect of longevity on the number of matings ($\chi^2 = 107.17$, d.f. = 1, $P < 0.001$), with longer-lived females mating more over their lifetime. We also found a significant negative effect of assay start date ($\chi^2 = 17.66$, d.f. = 1, $P < 0.001$). After controlling for longevity and temporal variation, we found a significant sire effect on the lifetime number of matings (Table 1). This measure of mating frequency exhibited substantial levels of additive genetic variance (V_A) and high evolvability (CV_A) and narrow-sense heritability (Table 1). Sire family means of the residuals from a linear regression of lifetime number of matings on longevity and assay start date are displayed in Fig. 1a.

Mating proportion also showed substantial phenotypic variation (mean ± SD = 0.25 ± 0.11 range = 0.05–1.00, Fig. 1b). Longevity had a significant effect ($z = -6.62$, $n = 625$, $P < 0.001$), with longer-lived females having a lower mating proportion. After controlling for longevity, we found a significant sire effect for mating proportion, which also exhibited substantial levels of additive genetic variance and high evolvability and narrow-sense heritability (Table 1).

In contrast, P_2 exhibited low and nonsignificant heritability and low levels of additive genetic variance and evolvability (Table 1). Although there was phenotypic variation among males in P_2 (mean ± SD = 0.76 ± 0.25; range = 0.07–1, Fig. 1c), we found nonsignificant variance among sire families when controlling for the significant positive effect of egg-to-adult viability ($z = 2.619$, $n = 205$, $P = 0.008$). Sire family means of the residuals from a logistic regression of P_2 on egg-to-adult viability and date of mating are displayed in Fig. 1c. Variance estimates calculated on the logit scale for P_2 did not differ from estimates on the raw scale and can be found in supplementary material (Table S1). There was no genetic correlation between residual P_2 and residual female mating proportion ($r = 0.064$, d.f. = 49, $P = 0.657$, Fig. 2).

Table 1 Quantitative genetic parameters for female lifetime number of matings, female lifetime mating proportion, P_2 and egg-to-adult viability.

	N	Mean (SE)	n sires	n dams	V_{Sire} (SE)	V_{Dam} (SE)	V_A (SE)	V_P (SE)	V_R (SE)	h^2 (SE)	CV_A (SE)	CV_P (SE)	CV_R (SE)	I_A (SE)	P_{Sire}	P_{Dam}
Lifetime number of matings	625	3.592 (0.641)	70	197	0.274 (0.096)	0.019 (0.090)	1.095 (0.382)	2.148 (0.156)	1.854 (0.182)	0.510 (0.168)	0.291 (0.054)	0.408 (0.016)	0.286 (0.045)	0.085 (0.031)	< 0.001*	0.95
Lifetime mating proportion	625	0.256 (0.005)	70	197	0.002 (< 0.001)	0 (< 0.001)	0.006 (0.002)	0.011 (0.001)	0.009 (0.001)	0.589 (0.136)	0.327 (0.042)	0.427 (0.020)	0.274 (0.046)	0.107 (0.028)	< 0.001*	1
P2	205	0.764 (0.018)	53	126	0.001 (0.003)	0.003 (0.006)	0.002 (0.011)	0.062 (0.007)	0.059 (0.009)	0.037 (0.182)	0.063 (0.189)	0.325 (0.026)	0.319 (0.041)	0.004 (0.019)	0.472	0.251
Egg-to-Adult viability	205	0.772 (0.170)	53	128	0.009 (0.006)	0.005 (0.004)	0.036 (0.026)	0.052 (0.008)	0.038 (0.007)	0.685 (0.466)	0.244 (0.098)	0.295 (0.029)	0.166 (0.109)	0.060 (0.044)	0.157	0.029*

*Significant P values ($P < 0.05$).
Number of offspring (N), trait means, number of sire (half-sib) and dam (full-sib) families (n), variance components for sires (V_{Sire}) and dams (V_{Dam}), additive genetic variation (V_A), total phenotypic variation (V_P), residual variation (V_R), narrow-sense heritabilities (h^2), mean-standardized additive genetic variances (evolvabilities: CV_A and I_A), coefficient of phenotypic variation CV_P, coefficient of residual variation CV_R and significance values for sire and dam effects (P_{Sire} and P_{Dam}). Standard errors (SE) are provided within brackets.

Egg-to-adult viability displayed considerable phenotypic variation (mean ± SD = 0.77 ± 0.25) but low levels of additive genetic variance (Table 1) and non-significant variance among sires ($P = 0.165$) after controlling for a positive effect of assay date on egg-to-adult viability ($z = 4.709$, $n = 205$, $P < 0.001$). The raw sire family means for measures of female mating frequency and P_2 are provided in Figure S1.

Discussion

Understanding the adaptive significance of polyandry has been a central goal in sexual selection research over the past three decades. One explanation for the maintenance of polyandry embodied by the 'sexy sperm' and 'good sperm' hypotheses proposes that sperm competition may play a role in the evolution of polyandry (Keller & Reeve, 1995; Yasui, 1997). These hypotheses have been the subject of considerable debate (Pizzari & Birkhead, 2002), and a recent theoretical analysis suggested that sexy sperm processes are unlikely to favour the evolution of polyandry when mating imposes costs (Bocedi & Reid, 2014). Nonetheless, the debate is one that is only likely to be resolved empirically. We provide a quantitative genetic approach to investigate the variation within a population to test the assumptions of the sexually selected sperm models.

Heritability of polyandry

We found evidence for substantial additive genetic variance in polyandry and report high estimates of evolvability (CV_A and I_A), suggesting that polyandry can respond to selection. A key strength of our experimental methodology was the use of a comprehensive measure of female mating frequency on a large sample size, giving females multiple opportunities to mate for the entire duration of their lifespan. Arguably, this measure of mating frequency gives a more accurate estimate of polyandrous behaviour compared to a single point measure of female mating frequency used in the majority of previous studies (Fukui & Gromko, 1991; Simmons, 2003; Shuker *et al.*, 2006; Evans & Gasparini, 2012; Arbuthnott *et al.*, 2014). For example, our measure is free of any age-specific variation in female receptivity to mating that might affect point estimates.

Our experiment gave females sequential mating opportunities three times a week. We did not expose females to multiple males in mating opportunities to prevent male–male competition contributing to the variation in female mating rates. In the wild, females may have numerous males to choose from at any given time. Therefore, our experimental design did not exactly mimic how females would be exposed to potential mates in the wild. However, in *Drosophila pseudoobscura*, previous work has shown that the variation of female remating in the laboratory (when exposed to a

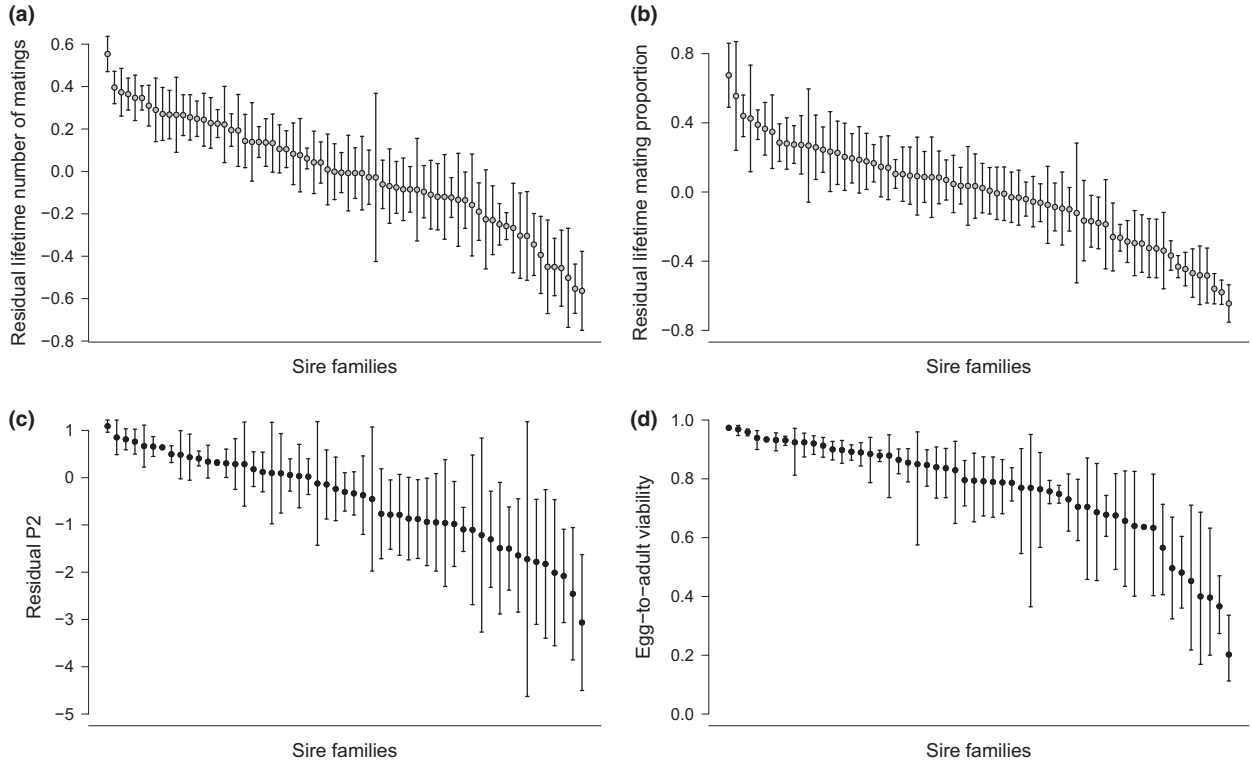

Fig. 1 Sire family mean ± SE of phenotypic traits after accounting for significant covariates on the trait of interest: (a) residual female lifetime number of matings, controlling for the effect of longevity and assay start date, (b) residual female lifetime mating proportion, controlling for the effect of longevity, (c) residual P_2, controlling for the effect of egg-to-adult viability, and (d) egg-to-adult viability controlling for the effect of assay start date. Residuals were obtained from a linear model (lifetime number of matings) or logistic regression models (lifetime mating, P_2, and egg-to-adult viability). Sire families were sorted by decreasing y values. Plots of the raw sire family means can be found in the Figure S1.

single male at a time) reflected rates of polyandry (assessed by multiple paternity) in the wild (Price *et al.*, 2011), suggesting that mating frequency measured in sequential mating opportunities provides a good estimate of polyandry in the wild.

Underestimates of additive genetic variation are likely to be reported in any trait that is at least partially determined by the phenotypes of other individuals, due to sampling variance arising from the use of random non-focal individuals (Garcia-Gonzalez & Evans, 2011). Male mating success (and female remating) in *D. melanogaster* is influenced by secondary sexual traits such as sex combs (Ahuja & Singh, 2008), cuticular hydrocarbons (Ferveur, 2005) and body size (Partridge & Farquhar, 1983; Partridge *et al.*, 1987; Bangham *et al.*, 2002). Males also influence female mating behaviour after copulation through the transfer of seminal fluid proteins. These proteins cause physiological changes that induce nonreceptivity in females after mating (Kalb *et al.*, 1993; Wolfner, 1997, 2002). Experimental evolution studies in *Drosophila* have found evidence for divergence in male ability to induce refractory periods in females (Rice, 1996; Holland & Rice, 1999; Pitnick *et al.*, 2001a), suggesting the presence of genetic variation in male ability to prevent female remating. Thus, variation between males in the expression of both pre- and post-copulatory traits may affect the rate of female mating. By using males from isogenic lines reared and housed in standard larval and adult densities, we aimed to minimize genetic and environmental variation attributable to mate identity that could introduce noise in the estimation of genetic variance in female-determined mating rates. Similarly, variation in nonadditive genotypic male x female interactions is expected to obscure the patterns of additive genetic variance (Moore *et al.*, 1997; Garcia-Gonzalez & Evans, 2011; Tennant *et al.*, 2014). To minimize the variation attributable to genetic compatibility, we used a wide range of male genotypes (10 isogenic lines) as mates, using a single isogenic line across all females within a single mating opportunity. However, stochastic variance due to male effects could not be completely eliminated as the order of male genotypes remained the same across mating opportunities. Therefore, if a female did not mate in any given

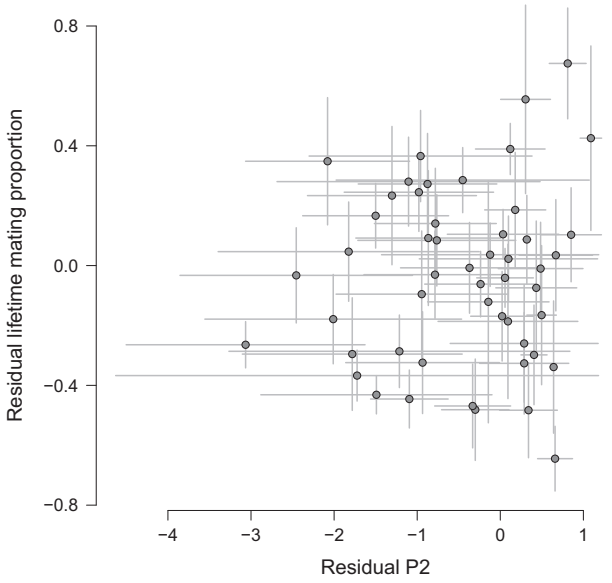

Fig. 2 Sire family mean relationship between residual P_2, controlling for the effect of egg-to-adult viability, and residual female lifetime mating proportion controlling for the effect of longevity. Residuals were obtained from logistic regression models. Points and error bars correspond to sire family means and standard errors, respectively.

opportunity, she was not presented with the same genotype again on the following opportunity, but with the genotype to which all other females were exposed. By keeping the order of male genotypes equal across mating opportunities, we may have introduced variance due to female age × male genotype interactions. Nevertheless, although imperfect, the controls we have implemented provide a relatively accurate estimate of additive genetic variance in female-determined mating rates in this species.

Previous evidence for genetic variance in female mating frequency in *D. melanogaster* comes indirectly from studies using experimental selection regimes. For example, female mating frequency responds to directional selection (Gromko & Newport, 1988; Fukui & Gromko, 1991; Torres-Vila *et al.*, 2002). Arbuthnott *et al.* (2014) found significant divergence in female remating among eight replicate populations evolving in novel environments, and Pitnick *et al.* (2001a) reported evolutionary divergence in female remating rate following experimental removal of sexual selection. Evolutionary responses to selection on female mating frequency are expected to be rapid, given the magnitude of additive genetic variance documented here. Our findings establish evidence for high levels of additive genetic variation in polyandry in a population recently derived from the wild. This finding supports the first assumption of the sexy and good sperm models that polyandry exhibits evolutionary potential.

Heritability of sperm competitiveness

The presence of additive genetic variation in sperm competitiveness is a key question in sexual selection research (Garcia-Gonzalez & Evans, 2011). For instance, the existence of heritable variation in the ability of males to fertilize eggs under sperm competition is a key assumption in both sexy and good sperm processes (Evans & Simmons, 2008). Contrary to this assumption, we found low and nonsignificant additive genetic variance in P_2 and no genetic covariation between P_2 and polyandry, which suggests that sexually selected sperm processes are unlikely to contribute to the evolution of polyandry in this population of *D. melanogaster*.

P_2 estimates could be influenced by copula duration of either the rival or focal male, which we did not measure in our study. However, in *D. melanogaster*, Gilchrist & Partridge (2000) show that the first half of copula duration is dedicated to sperm transfer, whereas the remaining time is given mainly to the transfer of seminal fluid proteins to prevent female remating. Their findings suggest no clear relationship between copulation duration and sperm transfer, suggesting that copula duration is not a strong predictor of paternity success in this species.

There is mixed evidence for the existence of genetic variance of P_2 in *D. melanogaster*. Arbuthnott *et al.* (2014) found no evidence for variation in P_2 between eight replicate populations of *D. melanogaster*. An experimental evolution study failed to generate divergence in defensive (P_1) or offensive (P_2) sperm competitiveness (Bjork *et al.*, 2007), and a previous quantitative genetic analysis of P_2 found little evidence for additive genetic variation (Hughes, 1997). However, Friberg *et al.* (2005) found small but significant levels of additive genetic variance in components of sperm offence and defence in hemiclones derived from a large outbred population of *D. melanogaster*. As a response to altering the strength of sexual selection by manipulating the operational sex ratio for 55–60 generations in a laboratory population of *D. melanogaster*, Nandy *et al.* (2013) found a decrease in P_2 in female-biased lines (male–male competition reduced), but failed to detect any difference between mean P_2 in male-biased lines (increased male–male competition) and control lines (balanced sex ratio). The latter findings are consistent with the possibility that P_2 ability was fixed in the ancestral population and that the decrease in P_2 observed in the female-biased treatment after 55–60 generations arose due to the accumulation of deleterious mutations that were not under purging selection. Thus, Nandy *et al.*'s (2013) findings cannot be taken as unequivocal evidence for the presence of standing additive genetic variation in P_2 in *D. melanogaster*. Our study used flies from a population recently derived from the wild. The lack of heritable variation found in our population may be

due to a history of intense directional selection on genes contributing to P_2 in the wild, resulting in fixation or near fixation of favourable alleles, leaving the population with low levels of additive genetic variation. It is also possible that some additive genetic variation in our population could have been eroded in the six generations from their introduction to the laboratory prior to the start of the experiment.

In other species, empirical evidence for genetic variation in P_2 does exist. In the yellow dung fly *Scathophaga stercoraria*, Hosken *et al.* (2001) found increased P_2 success as a response to experimental evolution under polyandry. Firman & Simmons (2011) found that male house mice from polyandrous experimental evolution lines had higher P_2 success than males from monandrous lines. The lines of mice used in these experiments had been derived from a population with a long history of enforced monogamy, so the strength of the observed response could have been inflated relative to natural populations, due to the reintroduction of post-copulatory sexual selection in these laboratory lines. Nonetheless, among-population variation in sperm competitiveness has been found among natural populations of house mice that differ in the levels of selection from sperm competition (Firman & Simmons, 2014).

It has been argued that fertilization success is not a perfect predictor of sperm competitiveness due to variance associated with the sampling of tester individuals (e.g. rival males or tester females), regardless of the existence of genotypic male–male or male–female interactions (Garcia-Gonzalez, 2008b). Empirical studies have emphasized the significance of male × male × female genotypic interactions (Clark & Begun, 1998; Clark *et al.*, 1999; Clark, 2002; Colegrave *et al.*, 2002; Bjork *et al.*, 2007) in determining the outcome of competitive fertilization success. In addition to genotypic interactions, male × male interactions can be a source of stochasticity if some males do better (or worse) against the average male in the population (Clark, 2002; Garcia-Gonzalez, 2008b; Garcia-Gonzalez & Evans, 2011). Genetic variance in sperm competitiveness will be underestimated when the variation in sperm competitive ability of randomly sampled rival males is not taken into account (Garcia-Gonzalez, 2008b; Garcia-Gonzalez & Evans, 2011). Therefore, sampling variance associated with male × male × female interactions can obscure estimates of intrinsic competitive fertilization success, which will lead to inaccurate estimates of its genetic basis.

Given these stochastic sources of variance, we attempted to minimize sampling variance by using individuals from isogenic lines. By doing so, we reduced variation in P_2 that arises due to genotypic and nongenotypic interactions. Yet still we found little evidence for additive genetic variance in P_2. One possible explanation for our finding could be our use of isogenic nonfocal individuals; we detected low levels of additive genetic variance in P_2 when males were mated to a specific female genotype and competed against a specific male genotype. The use of only one rival male genotype and one female genotype could result in failure to detect additive genetic variation in sperm competitiveness if a negative covariance between transitive sperm competitiveness and genotypic interaction effects existed (Engqvist, 2013). However, negative covariances between sperm competitiveness and interaction effects are unlikely to occur between all sire families and the isogenic lines. Generally, it is more likely that our experimental design would result in an overestimate of the true variance across sperm competitiveness, because the variance estimates include nonadditive interaction variance (Engqvist, 2013). Future studies could use rival males and females from a number of isogenic lines to account for genotypic male × male × female interaction effects in order to allow for estimations of the relative importance of additive effects and effects due to genotypic interactions.

The standardization of nonfocal individuals allows us to minimize sampling variance induced by randomly selecting females (and rival males) that differ in their overall effects. The choice of the tester homogeneous background is expected to determine the mean of the sperm competition phenotype. For instance, if the chosen rival male isogenic line is a genotype with low sperm competitiveness, the estimated mean of the population would be high and vice versa. However, it should not affect the magnitude of the variance in the trait within the population. We note that the mean P_2 phenotype reflects very closely that reported in previous studies of sperm competitiveness in *D. melanogaster* (Boorman & Parker, 1976; Fiumera *et al.*, 2005; Morrow *et al.*, 2005) so our homogenous background appears to be representative of the species generally. Despite the risk of inflated variance estimates from the use of isogenic nonfocal individuals, we found no additive genetic variation in P_2. Our findings thus suggest that there is insufficient additive genetic variation in sperm competitive ability to enable the evolution of polyandry through the sexy sperm process.

Heritability of egg-to-adult viability

Our genetic analysis of male ability to produce viable offspring revealed low levels of additive genetic variance and nonsignificant sire effects. Although heritability in egg-to-adult viability has previously been documented in *D. melanogaster* (Gilchrist & Partridge, 1997; Chippindale *et al.*, 2001), our estimate is the first to measure the males' ability to generate viable offspring independent of female effects. We found no evidence of intrinsic male ability to produce viable offspring. However, the significant dam effect on egg-to-adult viability that we report supports Friberg *et al.*'s (2011) finding of substantial X-linked effects on this

important fitness trait in *D. melanogaster*. A competitive egg-to-adult viability assay, where larvae from the offspring of the wild-type focal male competed against larvae from the rival male, would have been even better than the noncompetitive assay we used. However, noncompetitive egg-to-adult viability has been used previously to estimate genetic variance in the trait (e.g. Gilchrist & Partridge, 1997) and is normally taken as a valid proxy for egg-to-adult viability in broader contexts.

The significant positive effect of egg-to-adult viability on P_2 estimates supports Gilchrist & Partridge's (1997) findings that observed differences in P_2 among males can be strongly influenced by differential egg-to-adult viability and further highlights the importance of accounting for its effect when measuring genetic variation in P_2 and when testing good sperm processes (Garcia-Gonzalez, 2008a). However, the positive relati onship between egg-to-adult viability and P_2 estimates found here cannot be taken as support for a good sperm process, because neither of these traits harboured significant additive genetic variance, and the genetic correlation between P_2 and polyandry predicted by both the sexy and good sperm processes was also nonsignificant.

Conclusion

In conclusion, because polyandry holds such important ecological and evolutionary consequences (Pizzari & Wedell, 2013 and references therein), there has been considerable interest in determining the evolutionary potential of female mating frequency and in understanding the mechanisms that drive evolutionary change in female mating rates. Our finding of substantial additive genetic variation in female mating frequency strongly underpins the potential for direct and indirect selection to drive and constrain the evolution of polyandry. However, the lack of additive genetic variance in sperm competitiveness reported here suggests that polyandry may not be maintained via sexually selected sperm processes in this population.

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Data archive location

Data will be available from Dryad (http://datadryad.org/) upon acceptance for publication.

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Supporting information

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

Table S1 Quantitative genetic paramters for logit transformed P2.

Figure S1 Raw sire mean ± SE of (a) female lifetime number of matings, (b) female lifetime mating proportion and (c) P2.

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