Journal of Insect Physiology 60 (2014) 1–6

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

No inbreeding depression in sperm storage ability or offspring viability in *Drosophila melanogaster* females

Outi Ala-Honkola [a,b,*], Mollie K. Manier [b,c], Stefan Lüpold [b], Elizabeth M. Droge-Young [b], William F. Collins [b], John M. Belote [b], Scott Pitnick [b]

[a] Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014, Finland
[b] Department of Biology, Syracuse University, Syracuse, NY 13244, USA
[c] The George Washington University, Biological Sciences, 2023 G St. NW, Washington, DC 20052, USA

ARTICLE INFO

Article history:
Received 8 August 2013
Received in revised form 21 October 2013
Accepted 23 October 2013
Available online 1 November 2013

Keywords:
Inbreeding depression
Sperm storage
Male choice
Drosophila melanogaster

ABSTRACT

Mating between relatives usually decreases genetic quality of progeny as deleterious recessive alleles are expressed in inbred individuals. Inbreeding degrades sperm traits but its effects on sperm storage and fate within females are currently unknown. We quantified the relationship between the degrees of inbreeding relevant to natural populations ($f = 0$, 0.25 and 0.50) and the number of sperm inseminated and stored, sperm swimming speed, long-term sperm viability while in storage, pattern of sperm precedence, mating latency, and offspring viability of female *Drosophila melanogaster*. The use of transgenic flies that have either red or green fluorescent sperm heads allowed us to distinguish two ejaculates in the female reproductive tract and facilitated quantification of sperm storage and use traits. We found no inbreeding depression in either long- or short-term sperm storage ability. The most inbred females exhibited significantly longer mating latency, which could be explained by males preferring to mate with outbred females. On the other hand, as no evidence for cryptic male choice in the form of ejaculate tailoring of sperm number was found, the most inbred females might just be less eager to mate. We also found no evidence that the degree of maternal inbreeding influenced offspring viability. Comparison with a contemporaneous study of male inbreeding consequences for ejaculate quality suggests that inbreeding depression is more severe in males than in females in our study population.

1. Introduction

Mating between close relatives often leads to a decrease in fitness known as inbreeding depression (Lynch and Walsh, 1998). Due to human disturbance, many animal populations are becoming smaller and more fragmented, which increases the likelihood of mating between kin (Frankham et al., 2002). Under such circumstances, inbreeding depression can be strong enough to drive small populations to extinction (Saccheri et al., 1998; O'Grady et al., 2006) and therefore understanding the effects of inbreeding on various reproductive characters is becoming increasingly important. For example, several studies show that sperm number and quality (e.g., sperm motility or proportion of morphologically normal sperm in an ejaculate) are poor in inbred populations (Wildt et al., 1982; Roldan et al., 1998; Gomendio et al., 2000; Margulis and Walsh, 2002; van Eldik et al., 2006; Gage et al., 2006; Fitzpatrick and Evans, 2009), which may pose a risk to population persistence. However, nothing is known about how inbreeding affects female reproduction at the level of sperm storage and handling, most likely due to the challenge of studying sperm storage *in vivo*. Female sperm storage traits may, however, affect reproductive success of threatened populations if, for example, long-term sperm storage is compromised and remating opportunities to replenish sperm reserves are limited. Having an inbred mother may also be costly for an offspring that is itself outbred due to compromised maternal care as has been shown in mice (White, 1972) and the burying beetle (*Nicrophorus vespilloides*; Mattey et al., 2013) but not in red deer (*Cervus elaphus*; Walling et al., 2011). More knowledge on cross-generational inbreeding effects is needed for conservation purposes, for example in decisions of

Abbreviations: AR1, autocorrelation structure of order 1; GFP, green fluorescent protein; GLS, generalised least squares; LMM, linear mixed model; L-ratio, likelihood ratio; ML, maximum likelihood; PBS, phosphate buffered saline; P_2, proportion of offspring sired by the second male to mate; REML, restricted maximum likelihood; RFP, red fluorescent protein; SOM, supplementary online material; SPTH, spermathecae; SR, seminal receptacle.

* Corresponding author at: Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014, Finland. Tel.: +358 400815674; fax: +358 142601021.

E-mail addresses: outi.alahonkola@gmail.com, outi.ala-honkola@jyu.fi (O. Ala-Honkola), maniermk@gmail.com (M.K. Manier), sklupold@syr.edu (S. Lüpold), emrdoge@syr.edu (E.M. Droge-Young), wfcollin@gmail.com (W.F. Collins), jbelote@syr.edu (J.M. Belote), sspitnic@syr.edu (S. Pitnick).

0022-1910/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jinsphys.2013.10.005

whether or not to use inbred mothers in captive breeding programs.

The relative attractiveness of inbred *versus* outbred females to males may affect the mating success of inbred females. Males may prefer to mate or invest more resources to outbred females if mating with an inbred female poses a risk of fitness loss due to the negative effects of maternal inbreeding on offspring production (e.g., Richardson et al., 2004; Mattey et al., 2013). This question has only been studied in the mealworm beetle *Tenebrio molitor* (Pölkki et al., 2012), but no preference for outbred females was found. There are several ways to measure attractiveness in *Drosophila*. First, attractive males mate sooner when together with the female (Barth et al., 1997; Ritchie et al., 1999; Taylor et al., 2008 and see Nandy et al., 2012 for a study of female attractiveness). Second, longer copulations indicate a bigger investment (i.e., preference) to a given female (Friberg, 2006; Bretman et al., 2009), assuming that copulation duration is under male control (e.g., Jagadeeshan and Singh, 2006) and third, greater number of sperm ejaculated indicates a bigger investment (e.g., Lüpold et al., 2011).

The aim of this study was to experimentally vary the level of inbreeding of *Drosophila melanogaster* (with treatment variation relevant to the structure of natural populations; i.e., $f = 0$, 0.25 and 0.50) and then to quantify variation in traits important to female and offspring fitness, including measures of female attractiveness (i.e., mating latency, copulation duration and male ejaculate expenditure), long-term sperm viability in female reproductive tract, sperm storage, sperm swimming speed, and sperm competition dynamics (i.e., proportion of offspring sired by the 2nd male to mate, P_2), in addition to offspring number and viability. The inbreeding coefficient (f) is calculated as $f_t = \frac{1}{4}(1 + 2f_{t-1} + f_{t-2})$ (Falconer and Mackay, 1996 p. 89), assuming $f = 0$ in the base population. The effects of inbreeding on several male reproductive traits were assayed contemporaneously in these same lines and have been reported in Ala-Honkola et al. (2013). Therefore, we were also able to compare the severity of inbreeding depression between the two sexes. The use of transgenic flies that express either red or green fluorescent sperm heads (Manier et al., 2010) allowed us to distinguish between the ejaculates of two males in competition and to quantify *in vivo* sperm motility and sperm numbers at various locations and times following insemination within the selective environment of the female reproductive tract (Manier et al., 2010, 2013; Lüpold et al., 2011, 2012, 2013).

2. Materials and methods

2.1. Experimental material

The experimental flies originated from a line genetically engineered to produce sperm tagged with a red fluorescent protein (RFP; DsRed-Monomer) that has been backcrossed for six generations to the LHM wild-type strain (for details on the fly strains and the genetic transformation methods, see Manier et al., 2010). We generated lines of flies that differed in inbreeding coefficient by mating full-siblings for either zero (outbred control lines), one (moderately inbred lines, $f = 0.25$), or three generations (highly inbred lines, $f = 0.5$), following Zajitschek et al. (2009); Fig. 1; see also Ala-Honkola et al., 2013). To generate the outbred control lines, a female from a given line was mated to a male from a randomly selected outbred line. All lines (with one back-up for each line) were created from 60 full-sibling families (F_0) founded by placing pairs of randomly selected virgin females and males from the RFP-line into plastic 8-dram vials containing cornmeal–molasses–agar–yeast medium (5.4% cornmeal, 7% molasses, 0.5% agar, 2% yeast, 1.2% ethanol, 0.4% propionic acid, 0.06% methylparaben added to water) and a few grains of live yeast. In F_4, we had 56 lines

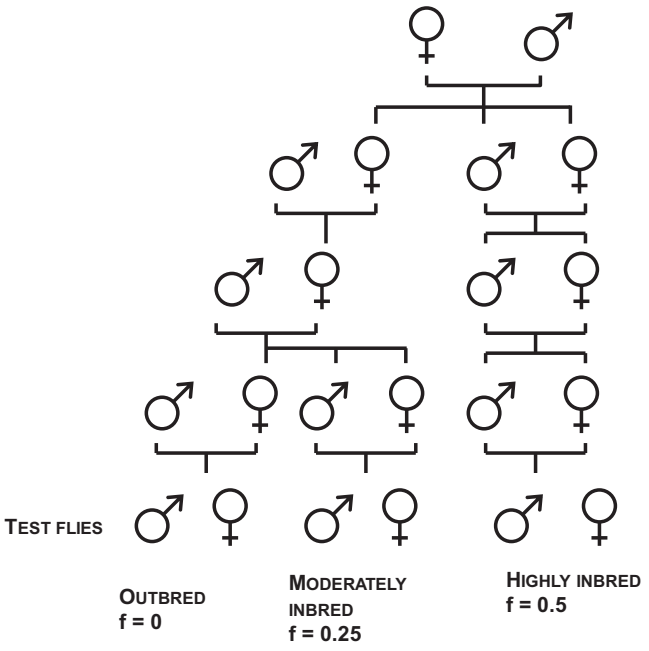

Fig. 1. Breeding design to create flies with different inbreeding coefficients.

in each treatment. During culturing, each pair was transferred to a new vial three times a week to avoid larval crowding. Virgin females and males for the experiments were collected under CO_2 anesthetisation.

When testing the effects of inbreeding on female traits, females were mated to outbred RFP males and/or to males of an outbred GFP/ubiquitin line that expresses green fluorescent protein in sperm heads and a ubiquitin green fluorescent body marker. The ubiquitin body marker allows unambiguous paternity assignment based on fluorescent body. All males were bottle reared and collected as virgins under CO_2 anesthetisation.

All experimental flies were 3–5 days old at their first experimental mating. In order to remove variation in P_2 and sperm traits attributable to the male's mating history (Bjork et al., 2007), each male was initially mated to a non-experimental virgin female one day before their first experimental mating. After the experiments, we measured thorax length for all females and males to the nearest 0.01 mm using the reticule of a dissection stereoscope (magnification 80×).

2.2. Single-mating productivity (i.e., long term sperm storage)

The potential effects of inbreeding on long-term sperm storage ability of females were estimated via offspring production over ten days after a single mating. We mated one virgin female from each inbred line to either a GFP/ubiquitin or an RFP line male (half of the lines were mated to GFP males and the other half to RFP males) and transferred females to fresh vials every day for ten days. Possible inbreeding depression in the egg-to-adult viability of the offspring was estimated from eggs laid on days 1, 3 and 5 by counting the numbers of eggs laid and the number of eclosed adults per vial (Droge-Young et al., 2012).

2.3. Mating latency, copulation duration, number of sperm ejaculated, sperm storage and P_2

An experiment to quantify the effects of female inbreeding on (1) mating latency, (2) copulation duration, (3) the number of sperm transferred, (4) the number of sperm females stored and

(5) the proportion of offspring sired by the second male to mate (P_2) was conducted as follows. On day 0, each virgin female was mated to either a GFP/ubiquitin or an RFP line male; on day 2 females were given a 4-h opportunity to remate with a male with a reciprocal sperm tag (i.e., RFP or GFP/ubiquitin). Females failing to remate on day 2 were provided similar opportunities on days 3–5. We counted all the adult progeny produced prior to remating (results reported for P_2 only) to ensure the first mating was successful and to estimate sperm usage of first-male sperm prior to remating. All matings were performed in one large replicate, having one inbred female per line in each experiment (i.e. sperm ejaculated, sperm stored and P_2). Pairs were aspirated into fresh plastic vials with 10 ml of medium and observed continuously, and mating latencies (time from the introduction of the pair into the vial until the start of the copulation), copulation durations and remating day were recorded for all matings.

The number of sperm ejaculated by males and stored by females was determined by quantifying all sperm present in the bursa, seminal receptacle (SR) and spermathecae (SPTH) within females that were flash-frozen in liquid nitrogen 60 min and 5 h after the second copulation started, respectively. These time-points were chosen because sperm storage peaks at 60 min, and most females have ejected excess second-male and displaced first-male sperm by 5 h after the start of mating (Manier et al., 2010). P_2 was calculated from offspring produced during 6 days after remating. These females were transferred to fresh vials after 1 and 3 days, frozen after 6 days and stored in −20 °C until dissection. This approach yielded P_2 values for day 1, days 2–3 and days 4–6, and allowed us to examine possible time trends in P_2.

For sperm counts, we dissected the female reproductive tract into a drop of phosphate-buffered saline (PBS) on a microscope slide and unfolded the seminal receptacle (SR) before covering the specimen with a coverslip and sealing it with paper cement. Sperm were counted under a fluorescent microscope at a magnification of 400× (see Manier et al., 2010 for a more detailed description). All sperm counts were done blind to the treatment. The number of sperm ejaculated was the total number of the second males' sperm in female reproductive tract in the 60 min freezing point. For the number of sperm stored we report both the number of second-male sperm in the SR and the number of sperm in the SR and the two SPTH with ducts combined in the 5 h freezing point. We dissected 20–30 females per treatment in each experiment.

2.4. Sperm swimming speed

In vivo sperm swimming speed was recorded in reproductive tracts of females once mated to RFP males ($N = 20$ per inbreeding treatment). Females were anesthetised with CO_2 120 min after the start of the copulation and their reproductive tracts were removed as described above and mounted under a coverslip in 20 μl (to standardise tract compression) of Grace's Supplemented Insect Medium (Invitrogen) at room temperature. Ten second-long movies (74 frames; AVI) were recorded within 3–7 min of anesthetisation using an Olympus DP71 digital camera and DP Controller Software version 3.3.1.292 (Olympus America Inc.).

Each movie was imported into NIH ImageJ (v. 1.42q, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) as a monochrome stack, which was then inverted from a dark background to a light background. We measured slice-by-slice instantaneous linear velocities (μm/s) for 10 sperm per male using the Manual Tracking plugin for ImageJ (available at http://rsbweb.nih.gov/ij/plugins/index.html). The total number of sperm within the entire SR was quantified to statistically control sperm velocity measures for density effects (Manier et al., 2010; Lüpold et al., 2012). Average instantaneous velocities were calculated per tracked sperm.

2.5. Statistical analysis

We used R 2.15.2 for statistical analysis (R Development Core Team, 2012). The standardised coefficient of inbreeding, δ, was calculated by dividing the difference in mean trait values between outbred and inbred individuals by the mean trait value of outbred individuals (Lande and Schemske, 1985).

Single-mating productivity, offspring viability and P_2 data consisted of repeated measures of the same individuals at regularly spaced time points. These data were analysed with generalised least squares (GLS) models (function *gls* in the library *nlme* in R). Only complete time series were included in the analyses. Females not producing any offspring after the first mating or having a P_2 value of "0" were excluded from the P_2 analyses because these occurrences are symptomatic of unsuccessful insemination.

Female and male thorax lengths, male type (GFP or RFP) and treatment (inbreeding level) × time interaction were entered as fixed factors into the full models. In the analysis of P_2, only the second males' thorax was measured and used as a covariate. The number of progeny produced before remating was also entered as a covariate as it estimates the number of first-male sperm used, which can influence P_2 (Ala-Honkola et al., 2011). We tested different variance–covariance structures between observations from the same individuals (compound symmetry, first order autoregressive (AR-1) and AR-1 with heterogeneous variances) and chose the one that best fit the data based on AIC values (see Statistical Computing Seminars, 2013, Repeated measures analysis with R: http://www.ats.ucla.edu/stat/r/seminars/Repeated_Measures/repeated_measures.htm and Diggle et al., 2009). P_2 values were arcsine square-root transformed as they are proportions.

The optimal fixed structure of the models was determined by comparing nested models using likelihood ratio tests (maximum likelihood, ML). The final model was refitted with restricted maximum likelihood (REML) estimation as suggested by Zuur et al. (2009). Models were validated by examining the homogeneity and independence of errors.

We used general linear (function *lm* in R) models to analyse the effect of inbreeding on sperm numbers and progeny production before and after remating in the P_2 experiment. Whenever male or female thorax lengths were found not to be correlated with the dependent variable, they were removed from the models. Thus, we typically fit treatment and male type as factors into our models.

Linear mixed models (LMM; function *lme* in library *nlme* in R) were used in analyses of mating latency, remating latency, copulation duration (line as a random factor in these analyses) and sperm swimming speed (male as a random factor). Male and female thorax lengths, male type and treatment were entered as fixed factors into the full models. For analyses of sperm swimming speed, the number of sperm in the SR was entered as a covariate. The optimal fixed structure of the models was determined as above.

3. Results

3.1. Long-term sperm storage, egg-to-adult viability and progeny production

The long-term sperm storage ability of females showed no inbreeding depression. Specifically, inbred females did not deplete stored sperm any faster than the outbred females during the ten-day offspring production experiment (i.e., treatment × time interaction for offspring production was not significant: likelihood ratio (L-ratio) = 21.78, $df = 18$, $p = 0.24$, see also Fig. 2). Also, there was no inbreeding depression in cumulative offspring production (L-ratio for treatment = 2.10, $df = 2$, $p = 0.35$, Fig. 2) or in offspring viability (L-ratio for treatment = 2.62, $df = 2$, $p = 0.27$, Fig. 3).

Egg-to-adult viability decreased as female thorax length increased (see final models in Table 1 and Table S1 in Supplementary online material, SOM).

3.2. Short term sperm storage, P_2 and sperm swimming speed

Inbred females stored equal amounts of sperm in their SR (F(treatment)$_{2,74}$ = 0.34, p = 0.71; F(male type)$_{1,74}$ = 1.07, p = 0.30) and SR and SPTH combined (F(treatment)$_{2,74}$ = 1.03, p = 0.36; F(male type)$_{1,74}$ = 2.00, p = 0.16; Table 2) as compared to outbred females. Our observed differences between inbred and outbred females were about 10–13% in the opposite direction than predicted (i.e., inbred females stored more sperm than the outbred ones) and it is therefore unlikely that we would have missed detecting inbreeding depression in these traits. In addition, the female inbreeding level had no effect on sperm competition dynamics. Specifically, neither the time × treatment interaction of P_2 (L-ratio = 3.1, df = 4, p = 0.54) nor P_2 itself differed among inbreeding levels (L-ratio = 0.002, df = 2, p = 0.999; see also Tables 2 and 3). Consistent with the results of the single-mating productivity experiment (see above), there was no inbreeding depression in the number of progeny produced prior to remating in the P_2 experiment (Table 2; F(treatment)$_{2,109}$ = 0.40, p = 0.67) or after remating (Table 2; F(treatment)$_{2,105}$ = 1.56, p = 0.22). Progeny production prior to remating was correlated with female size so that larger females produced more offspring (F(female thorax length)$_{1,109}$ = 8.21, p = 0.005). *In vivo* sperm swimming speed was not affected by the female inbreeding level (L-ratio = 2.7, df = 2, p = 0.26; Tables 2 and 3).

3.3. Effects of female inbreeding on attractiveness as mate

Highly inbred females took longer to mate (δ = −0.53) as compared to outbred controls. There was no difference in mating latency, however, between moderately inbred and control females (L-ratio for treatment = 12.9, df = 2, p = 0.0016; outbred vs. moderately inbred: Tukey p = 0.40; outbred vs. highly inbred: Tukey p < 0.001; moderately inbred vs. highly inbred: Tukey p = 0.058; see also Tables 2 and 3). No such effect was found for remating interval (L-ratio = 2.0, df = 2, p = 0.37) or for the duration of first (L-ratio = 3.7, df = 2, p = 0.15) and second (L-ratio = 5.1, df = 2, p = 0.08; Tables 2 and 3) matings, respectively. We further found no evidence for differential ejaculate expenditure by males as the number of sperm inseminated did not differ among female inbreeding levels F(treatment)$_{2,62}$ = 1.90, p = 0.16; Table 2).

3.4. Performance differences between males from RFP and GFP lines

Although it was not a main interest of this study, we noticed that our RFP and GFP line males differed from each other in many respects. Generally, RFP males were "better" than GFP males, as offspring production was higher when females mated with RFP males both in the single-mating productivity (see Table S1 in SOM) and the P_2 experiments (when females mated first with RFP males in the P_2 experiment, they produced more offspring before the second mating (F(male type)$_{1,109}$ = 35.0, p < 0.001), and when the second mate was an RFP male, offspring production after the second mating was higher (F(male type)$_{1,104}$ = 6.14, p = 0.015)). Additionally, offspring viability was higher for females mated to RFP males (Table 1), and RFP males were better sperm competitors than GFP males (P_2 was higher when RFP males mated in the role the 2nd male; L-ratio for male type = 36.7, df = 1, p < 0.0001; see also Table 3). Copulations with RFP males were shorter than with GFP males but mating latency was longer for RFP males whereas remating speed was shorter (Table 3). Ejaculates of GFP males were larger than those of RFP males (F(male type)$_{1,62}$ = 33.6, p < 0.001), which is probably at least partly explained by the fact that green sperm are more easily detectable than red sperm due to a stronger fluorescent signal.

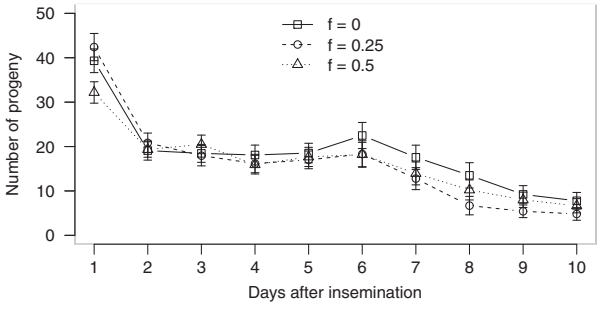

Fig. 2. Number of progeny (mean ± SE) females with different inbreeding levels produced during 10 days after a single mating with an outbred male; N = 41 in f = 0, N = 40 in f = 0.25 and N = 39 in f = 0.5.

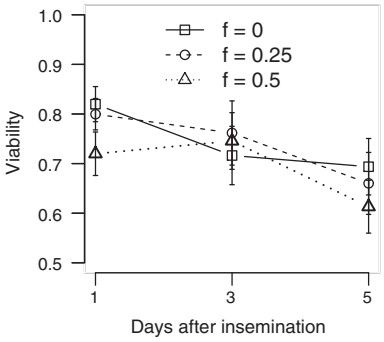

Fig. 3. Egg-to-adult viability (mean ± SE) of progeny when mothers differ in inbreeding coefficient; N = 34 in f = 0, N = 31 in f = 0.25 and N = 34 in f = 0.5.

Table 1
Final generalised least squares (GLS) model for offspring viability with first order autoregressive (AR1) correlation structure and male type (GFP/RFP) as a variance covariate. Intercept = male type GFP. Residual df = 294.

Fixed effect	Parameter estimate	SE	t	p
Intercept	1.79	0.369	4.88	<0.000
Female thorax length	−0.015	0.005	−3.14	0.002
Male type (RFP)	0.118	0.046	2.59	0.010

4. Discussion

We found almost no inbreeding depression in female reproductive traits. The only trait that showed inbreeding depression was mating latency of highly inbred females (δ = 0.53). Specifically, there was no inbreeding depression in sperm storage traits, neither in the short-term storage measured five hours after the second mating, nor in the long-term sperm storage measured indirectly as offspring production over ten days after a single mating. Similarly, female inbreeding had no effect on sperm swimming speed or on the pattern of sperm precedence. These results indicate that inbreeding did not influence those reproductive tract conditions critical for sperm storage and viability in our study population.

Even though fertility and offspring viability often show inbreeding depression in *D. melanogaster* (reviewed in Lynch and Walsh, 1998 and Enders and Nunney, 2011), our finding of no inbreeding depression in female fertility is not exceptional. Enders and Nunney (2011) did not find inbreeding depression in early progeny production (measured over 8 days; f = 0.25), which closely

Table 2
Effects of inbreeding on measured traits.

Trait	Mean ± SD (*N*)		
	Outbred, $f = 0$	Moderately inbred, $f = 0.25$	Highly inbred, $f = 0.5$
2nd-male sperm stored in SR	223 ± 102 (27)	240 ± 89 (25)	242 ± 99 (26)
2nd-male sperm stored in SR and SPTH	322 ± 131 (27)	365 ± 132 (25)	361 ± 119 (26)
P_2	0.85 ± 0.20 (39)	0.86 ± 0.17 (41)	0.86 ± 0.19 (34)
Progeny production before remating	121 ± 59.0 (39)	110 ± 60.6 (41)	111 ± 59.3 (34)
Progeny production after remating	167 ± 58.0 (38)	157 ± 64.1 (39)	142 ± 63.8 (32)
Sperm swimming speed (μm/s)	44.3 ± 18.6 (20)	50.3 ± 18.8 (16)	47.3 ± 19.5 (18)
Mating latency (min)	50.5 ± 50.4 (142)	58.7 ± 61.1 (136)	77.2 ± 73.9 (121)
Remating day	4.0 ± 0.88 (140)	3.9 ± 1.0 (130)	4.1 ± 1.0 (121)
Copulation duration in 1st mating (min)	27.9 ± 7.6 (141)	26.3 ± 7.1 (135)	26.9 ± 7.7 (120)
Copulation duration in 2nd mating (min)	32.7 ± 7.4 (140)	32.6 ± 9.4 (130)	30.9 ± 7.1 (121)
Ejaculate size	939 ± 422 (23)	1150 ± 415 (19)	1060 ± 469 (24)

Table 3
Final generalised least squares (GLS) model for the proportion of offspring sired by the second male to mate (P_2) and final linear mixed models for sperm swimming speed, mating latency, remating day and copulation duration. Intercept = outbred control lines and GFP males.

Trait	Fixed effect	Parameter estimate	SE	*df*	*t*	*p*	Random effect
P_2 (arcsine sqrt transformed)	Intercept	1.13	0.03	340	33	<0.001	
	2nd male type (RFP)	0.30	0.05	340	6.57	<0.001	
Sperm swimming speed	Intercept	58.6	5.28	484	11	<0.001	Male
	Number of sperm in the SR	−0.04	0.02	52	−2.3	0.027	
Mating latency ($\log_{10}$-transformed)	Intercept	1.44	0.04	233	34	<0.001	Line
	Highly inbred lines	0.19	0.05	162	3.58	0.0005	
	Moderately inbred lines	0.06	0.05	162	1.30	0.20	
	1st male type (RFP)	0.13	0.04	233	3.03	0.003	
Remating day	Intercept	4.35	0.07	225	66	<0.001	Line
	2nd male type (RFP)	−0.72	0.09	22	−7.85	<0.001	
Copulation duration with 1st male	Intercept	28.5	0.53	231	54	<0.001	Line
	1st male type (RFP)	−3.06	0.78	231	−3.90	<0.001	
Copulation duration with 2nd male	Intercept	34.9	0.56	225	62	<0.001	Line
	2nd male type (RFP)	−5.23	0.77	225	−6.84	<0.001	

corresponds to our single-mating productivity experiment (10 days). One could argue that we found no inbreeding depression in this study because our base population was already highly inbred. However, the base population has been maintained in a population cage with approximately 1000 individuals since its inception, and contemporaneous studies using isogenic lines derived from the same population to quantify and partition genetic variation in the same traits as examined here have documented extensive genetic variation in these traits (Lüpold et al., 2012, 2013).

Inbreeding depression in mating latency of highly inbred females could be explained either by male choice (i.e., males discriminating against highly inbred females) or by highly inbred females being less eager to mate. The male choice hypothesis is not supported, however, by data on the number of sperm inseminated. Males of our study population have previously been shown to tailor the size of their ejaculates based on female mating status, fecundity and age (Lüpold et al., 2011). Here we found no evidence of differential ejaculate allocation or adjustment of copulation duration based on inbreeding level of females. It is unlikely that we missed ejaculate tailoring due to low statistical power, as the observed difference was to the opposite direction than predicted (i.e., more sperm ejaculated to inbred females). The result of no ejaculate tailoring is not surprising, however, given that inbred females were as fecund as outbred females, and the extent of maternal inbreeding did not influence offspring viability. Consequently, the mating latency results may be indicative of highly inbred females being less motivated to mate.

There is great interest in the possibility of sex differences in the level of inbreeding depression, because stronger selection on males (indicated by stronger inbreeding depression) than females has the potential to lower the deleterious mutation load in females, which would reduce the cost of sex (Kodric-Brown and Brown, 1987; Agrawal, 2001; Siller, 2001; Whitlock and Agrawal, 2009). Thus, sexual selection, which is typically stronger on males than females, would purge deleterious mutations from populations, assuming that these mutations affect the overall health and vigour and hence mating success of males (e.g., the genic capture hypothesis; Rowe and Houle, 1996; Tomkins et al., 2004). Data presented here and in Ala-Honkola et al. (2013) allow comparison of inbreeding depression between the sexes. Inbreeding depression was higher in males than females: mating latency, remating latency and sperm competition success all showed inbreeding depression in males, whereas only mating latency showed inbreeding depression in females. Moreover, the level of inbreeding depression in mating latency was lower for females as compared to males ($\delta = 53\%$ in highly inbred females compared to 87% and 65% for highly and moderately inbred males, respectively). Results from our studies are thus consistent with those of Brittnacher (1981), Miller and Hedrick (1993), Enders and Nunney (2010) and Mallet and Chippindale (2011), who also found stronger inbreeding depression in male than female *D. melanogaster*. Stronger inbreeding depression in males has also been found in house mice (*Mus domesticus*; Meagher et al., 2000) and red flour beetles (*Tribolium castaneum*; Pray and Goodnight, 1995) suggesting that selection on males may indeed be stronger than on females.

5. Conclusions

Our study suggests that inbred females store and utilise sperm as effectively as outbred females do. In addition, maternal inbreeding was not detrimental to zygote and larval viability suggesting that inbred mothers do not necessarily pose a risk to offspring that are outbred. Inbreeding depression in our study population was

stronger on males than females suggesting that selection may be more intense on males, which could help to purge deleterious mutations from populations and thus reduce the cost of sex.

Acknowledgements

This research was supported by grants from the Academy of Finland (135684 and 250999) and Ella and Georg Ehrnrooth's Foundation to OA-H and from the U.S. National Science Foundation (DEB-0814732, DEB-1021240 and DEB1145965) to SP, JMB, SL and MKM, and by a fellowship of the Swiss National Science Foundation (PBSKP3_130878) to SL. The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2013.10.005.

References

Agrawal, A., 2001. Sexual selection and the maintenance of sexual reproduction. Nature 411, 692–695.
Ala-Honkola, O., Manier, M.K., Lüpold, S., Pitnick, S., 2011. No evidence for postcopulatory inbreeding avoidance in *Drosophila melanogaster*. Evolution 65, 2699–2705.
Ala-Honkola, O., Hosken, D.J., Manier, M.K., Lüpold, S., Berben, K.S., Collins, W.F., Belote, J.M., Pitnick, S., 2013. Inbreeding reveals mode of past selection on male reproductive characters in *Drosophila melanogaster*. Ecol. Evol. 3, 2089–2102.
Barth, M., Hirsch, H., Heisenberg, M., 1997. Rearing in different light regimes affects courtship behaviour in *Drosophila melanogaster*. Anim. Behav. 53, 25–38.
Bjork, A., Starmer, W.T., Higginson, D.M., Rhodes, C.J., Pitnick, S., 2007. Complex interactions with females and rival males limit the evolution of sperm offence and defense. Proc. R. Soc. B, Biol. Sci. 274, 1779–1788.
Bretman, A., Fricke, C., Chapman, T., 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. Proc. R. Soc. B, Biol. Sci. 276, 1705–1711.
Brittnacher, J., 1981. Genetic variation and genetic load due to the male reproductive component of fitness in *Drosophila*. Genetics 97, 719–730.
Diggle, P., Heagerty, P., Liang, K., Zeger, S., 2009. Analysis of Longitudinal Data. Oxford University Press, Oxford.
Droge-Young, E.M., Manier, M.K., Lüpold, S., Belote, J.M., Pitnick, S., 2012. Covariance among premating, post-copulatory and viability fitness components in *Drosophila melanogaster* and their influence on paternity measurement. J. Evol. Biol. 25, 1555–1563.
Enders, L.S., Nunney, L., 2010. Sex-specific effects of inbreeding in wild-caught *Drosophila melanogaster* under benign and stressful conditions. J. Evol. Biol. 23, 2309–2323.
Falconer, D.S., Mackay, T.F.C., 1996. Introduction to Quantitative Genetics. Longman, London, U.K..
Fitzpatrick, J.L., Evans, J.P., 2009. Reduced heterozygosity impairs sperm quality in endangered mammals. Biol. Lett. 5, 320–323.
Frankham, R., Ballou, J.D., Briscoe, D.A., 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge, UK.
Friberg, U., 2006. Male perception of female mating status: its effect on copulation duration, sperm defence and female fitness. Anim. Behav. 72, 1259–1268.
Gage, M.J.G., Surridge, A.K., Tomkins, J.L., Green, E., Wiskin, L., Bell, D.J., Hewitt, G.M., 2006. Reduced heterozygosity depresses sperm quality in wild rabbits, *Oryctolagus cuniculus*. Curr. Biol. 16, 612–617.
Gomendio, M., Cassinello, J., Roldan, E., 2000. A comparative study of ejaculate traits in three endangered ungulates with different levels of inbreeding: fluctuating asymmetry as an indicator of reproductive and genetic stress. Proc. R. Soc. B, Biol. Sci. 267, 875–882.
Jagadeeshan, S., Singh, R.S., 2006. A time-sequence functional analysis of mating behaviour and genital coupling in *Drosophila*: role of cryptic female choice and male sex-drive in the evolution of male genitalia. J. Evol. Biol. 19, 1058–1070.
Kodric-Brown, A., Brown, J.H., 1987. Anisogamy, sexual selection, and the evolution and maintenance of sex. Evol. Ecol. 1, 95–105.
Lande, R., Schemske, D.W., 1985. The evolution of self-fertilisation and inbreeding depression in plants. I. Genetic models. Evolution 39, 24–40.
Lüpold, S., Manier, M.K., Ala-Honkola, O., Belote, J.M., Pitnick, S., 2011. Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity, and age. Behav. Ecol. 22, 184–191.
Lüpold, S., Manier, M.K., Berben, K.S., Smith, K.J., Daley, B.D., Buckley, S.H., Belote, J.M., Pitnick, S., 2012. How multivariate ejaculate traits determine competitive fertilisation success in *Drosophila melanogaster*. Curr. Biol. 22, 1667–1672.
Lüpold, S., Pitnick, S., Berben, K.S., Blengini, C.S., Belote, J.M., Manier, M.K., 2013. Female mediation of competitive fertilisation success in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 110, 10693–10698.
Lynch, M., Walsh, B., 1998. Genetics and Analysis of Quantitative Traits. Sinauer Associates Inc., Sunderland, MA, pp. 980.
Mallet, M.A., Chippindale, A.K., 2011. Inbreeding reveals stronger net selection on *Drosophila melanogaster* males: implications for mutation load and the fitness of sexual females. Heredity 106, 994–1002.
Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving mechanisms of competitive fertilisation success in *Drosophila melanogaster*. Science 328, 354–357.
Manier, M.K., Belote, J.M., Berben, K.S., Lüpold, S., Ala-Honkola, O., Collins, W.F., Pitnick, S., 2013. Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. Evolution 67–8, 2348–2362.
Margulis, S., Walsh, A., 2002. The effects of inbreeding on testicular sperm concentration in *Peromyscus polionotus*. Reprod. Fertil. Dev. 14, 63–67.
Mattey, S.N., Strutt, L., Smiseth, P.T., 2013. Intergenerational effects of inbreeding in *Nicrophorus vespilloides*: offspring suffer fitness costs when either they or their parents are inbred. J. Evol. Biol. 26, 843–853.
Meagher, S., Penn, D.J., Potts, W.K., 2000. Male–male competition magnifies inbreeding depression in wild house mice. Proc. Natl. Acad. Sci. USA 97, 3324–3329.
Miller, P., Hedrick, P., 1993. Inbreeding and fitness in captive populations – lessons from *Drosophila*. Zoo Biol. 12, 333–351.
Nandy, B., Joshi, A., Ali, Z.S., Sen, S., Prasad, N.G., 2012. Degree of adaptive male mate choice is positively correlated with female quality variance. Sci. Rep. 2, 447.
O'Grady, J.J., Brook, B.W., Reed, D.H., Ballou, J.D., Tonkyn, D.W., Frankham, R., 2006. Realistic levels of inbreeding depression strongly affect extinction risk in wild populations. Biol. Conserv. 133, 42–51.
Pölkki, M., Krams, I., Kangassalo, K., Rantala, M.J., 2012. Inbreeding affects sexual signalling in males but not females of *Tenebrio molitor*. Biol. Lett. 8, 423–425.
Pray, L., Goodnight, C., 1995. Genetic variation in inbreeding depression in the red flour beetle *Tribolium castaneum*. Evolution 49, 176–188.
R Development Core Team, 2012. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org.
Richardson, D., Komdeur, J., Burke, T., 2004. Inbreeding in the *Seychelles warbler*: environment-dependent maternal effects. Evolution 58, 2037–2048.
Ritchie, M., Halsey, E., Gleason, J., 1999. *Drosophila* song as a species-specific mating signal and the behavioural importance of Kyriacou & Hall cycles in *D. melanogaster* song. Anim. Behav. 58, 649–657.
Roldan, E., Cassinello, J., Abaigar, T., Gomendio, M., 1998. Inbreeding, fluctuating asymmetry, and ejaculate quality in an endangered ungulate. Proc. R. Soc. B, Biol. Sci. 265, 243–248.
Rowe, L., Houle, D., 1996. The lek paradox and the capture of genetic variance by condition dependent traits. Proc. R. Soc. B Biol. Sci. 263, 1415–1421.
Saccheri, I., Kuussaari, M., Kankare, M., Vikman, P., Fortelius, W., Hanski, I., 1998. Inbreeding and extinction in a butterfly metapopulation. Nature 392, 491–494.
Siller, S., 2001. Sexual selection and the maintenance of sex. Nature 411, 689–692.
Statistical Computing Seminars, 2013. Repeated measures analysis with R. UCLA: Academic Technology Services, Statistical Consulting Group. Available from http://www.ats.ucla.edu/stat/r/seminars/Repeated_Measures/repeated_measures.htm, accessed May 15, 2013.
Taylor, M.L., Wedell, N., Hosken, D.J., 2008. Sexual selection and female fitness in *Drosophila simulans*. Behav. Ecol. Sociobiol. 62, 721–728.
Tomkins, J., Radwan, J., Kotiaho, J., Tregenza, T., 2004. Genic capture and resolving the lek paradox. Trends Ecol. Evol. 19, 323–328.
van Eldik, P., van der Waaij, E., Ducro, B., Kooper, A., Stout, T., Colenbrander, B., 2006. Possible negative effects of inbreeding on semen quality in Shetland pony stallions. Theriogenology 65, 1159–1170.
Walling, C.A., Nussey, D.H., Morris, A., Clutton-Brock, T.H., Kruuk, L.E.B., Pemberton, J.M., 2011. Inbreeding depression in red deer calves. BMC Evol. Biol. 11, 318.
White, J., 1972. Inbreeding effects upon growth and maternal ability in laboratory mice. Genetics 70, 307–317.
Whitlock, M.C., Agrawal, A.F., 2009. Purging the genome with sexual selection: reducing mutation load through selection on males. Evolution 63, 569–582.
Wildt, D.E., Baas, E.J., Chakraborty, P.K., Wolfle, T.L., Stewart, A.P., 1982. Influence of inbreeding on reproductive performance, ejaculate quality and testicular volume in the dog. Theriogenology 17, 445–452.
Zajitschek, S.R.K., Lindholm, A.K., Evans, J.P., Brooks, R.C., 2009. Experimental evidence that high levels of inbreeding depress sperm competitiveness. J. Evol. Biol. 22, 1338–1345.
Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.S., 2009. Mixed Effects Models and Extensions in Ecology with R. Springer, New York, NY, pp. 574.