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No evidence for precopulatory inbreeding avoidance in *Drosophila melanogaster*

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Inbreeding depression can lead to the evolution of inbreeding avoidance before or after mating. However, despite widespread evidence of inbreeding depression, studies of inbreeding avoidance have generated different results across populations or species. These differences could potentially reflect the confounding effects of factors such as magnitude of inbreeding depression, sex, social familiarity, state of primary sexual receptivity and mating history. We examined the influence of these proximate factors on precopulatory inbreeding avoidance in a laboratory-adapted, outbred population of *Drosophila melanogaster*. We found a significant but low coefficient of inbreeding depression based on egg–adult viability measures. Controlling for sex-specific responses, familiarity, sexual receptivity and mating history, we found no evidence of precopulatory inbreeding avoidance. Mate choice of virgins was random with respect to relatedness and measurements of courtship frequency, mating latency and mating duration did not indicate any preference for unrelated partners. In fact, the only evidence for differential sexual behaviour in response to relatedness was that males first mated to unrelated females were significantly faster to remate with related females than with unrelated females. These results suggest that inbreeding avoidance may be limited in outbred populations of *D. melanogaster*, and fit theoretical predictions that inbreeding is not selected against in either sex when the coefficient of inbreeding depression is relatively low.

Theory predicts that inbreeding depression, caused by the expression of deleterious recessive alleles or loss of overdominance effects suffered by the offspring of closely related parents, can promote the evolution of mechanisms of inbreeding avoidance (Charlesworth & Charlesworth 1987; Marr et al. 2002). In structured populations in which dispersal is limited or occurs after mating, inbreeding can be avoided before copulation through the recognition and avoidance of kin as mating partners. Evidence of precopulatory inbreeding avoidance, however, varies across and even within species. Negative assortative mating with respect to relatedness (i.e. inbreeding avoidance) has been documented in several studies of both vertebrates (e.g. Dewsbury 1982; Bateson 1983; Penn & Potts 1999) and invertebrates (e.g. Smith & Ayasse 1987; Simmons 1991; Stuart & Herbers 2000). Other studies, however, have failed to demonstrate precopulatory inbreeding avoidance (e.g. Keller & Arcese 1998; Guevara-Fiore et al. 2010), while some have reported preferences for mating with kin, both in invertebrates (e.g. Schjørring & Jäger 2007; Schjørring 2009) and in vertebrates (e.g. Thünken et al. 2007, 2011).

There are several explanations that could potentially account for this incongruence. First, variation in the magnitude of inbreeding depression is predicted to influence the intensity of selection on inbreeding avoidance across species or populations (Frommen & Bakker 2006; Kokko & Ots 2006). Second, theory predicts that inbreeding avoidance is sex specific (Parker 1979; Kokko & Ots 2006; Puurtinen 2011). Because females typically invest more in a reproductive event than males, for intermediate levels of inbreeding depression, females might be selected to avoid inbreeding while males are selected to inbreed (Parker 1979; Pizzari et al. 2004; Facon et al. 2006; Kokko & Ots 2006). Therefore, it is important to tease apart male- from female-specific sexual responses to kin. Third, lack of consideration for the proximate mechanisms causing inbreeding avoidance may undermine the power of a study to test inbreeding avoidance. Common proximate mechanisms that mediate precopulatory kin recognition and avoidance include prior association (where kin discrimination is based on social familiarity) and phenotype matching (where recognition is based on self-referent cues; Holmes & Sherman 1982; Holmes 1986). The relative influence of familiarity and phenotypic similarity in kin recognition has been investigated in vertebrates (Tang-Martinez 2001) but relatively little is known about the mechanisms of kin recognition in invertebrates. In addition, female *Drosophila* might be more likely to mate with

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strangers than familiar males, suggesting that individual recognition and mate choice are modulated by social familiarity (Ödeen & Moray 2008). Therefore, it is important to examine the effect of social familiarity on kin recognition when investigating inbreeding avoidance. Fourth, variation in a female's state of primary sexual receptivity (initial receptivity before first mating) is also associated with female choosiness (e.g. Lynch et al. 2005). This might influence the degree of inbreeding avoidance as a virgin female that is kept sexually isolated for a longer period might discriminate less between related and unrelated males. Consistent with this, in *Drosophila melanogaster*, recently eclosed female virgins (1-day posteclosion) display lower sexual receptivity than virgins that are given more time to mature (2-day posteclosion; Manning 1967) and this difference presents a convenient method of investigating the effect of the female's receptivity on inbreeding avoidance. Finally, in many insects, including *D. melanogaster*, the male seminal proteins transferred during mating cause dramatic changes in females that alter subsequent reproductive behaviour (reviewed in Wolfner 2002; Chapman & Davies 2004). However, little is known about inbreeding avoidance in nonvirgins and how it is adjusted by previous mating experiences. Seldom have all these factors been considered by inbreeding avoidance studies, making it difficult to interpret the evolutionary significance of variation in inbreeding avoidance.

Although *D. melanogaster* is capable of long-distance movements (up to 10 km; Yamazaki et al. 1986; Coyne & Milstead 1987), natural populations are characterized by limited dispersal and a tendency towards aggregations in particular localities (Wallace 1970; McInnis et al. 1982), which can increase the probability of related individuals interacting and the risk of inbreeding. Although direct information on inbreeding in wild *D. melanogaster* is scarce, estimates of genetic load suggest there is some inbreeding caused by patchy distribution of resources and population substructuring (Danieli & Costa 1977; Nielsen et al. 1985; Alonso-Moraga et al. 1988). Early work suggested potential for inbreeding avoidance in laboratory strains of *D. melanogaster*, by showing that in inbred isofemale lines flies avoid mating with individuals from the same line (Averhoff & Richardson 1974, 1976). However, a subsequent study failed to replicate these early results (van den Berg et al. 1984). In addition, studies investigating the role of relatedness in sexual behaviour in *D. melanogaster* have exclusively used virgin individuals (Averhoff & Richardson 1974, 1976; Mack et al. 2002), raising questions as to what extent these findings can be extrapolated to mated individuals: an important issue given that in most sexually reproducing organisms, including *D. melanogaster*, males and females typically mate multiply. Resolving patterns of inbreeding avoidance in *D. melanogaster* is therefore important in order to characterize a key aspect of the biology of the model organism, and more generally, to gain insight into the mechanisms underpinning precopulatory inbreeding strategies in invertebrates.

We addressed these goals in a laboratory-adapted outbred population of *D. melanogaster*. We first examined whether inbreeding depression occurred in our population. We then tested for evidence of sex-specific inbreeding avoidance behaviours via no-choice and mate choice assays and examined the proximate influence of social familiarity, primary receptivity and mating history.

METHODS

Experimental Population and Culturing

For all experiments we used a laboratory-adapted Dahomey wild-type stock of *D. melanogaster*. Flies were maintained at 25 °C, in a nonhumidified room, on a 12:12 h light:dark cycle, and fed standard sugar–yeast–maize–molasses medium with excess live yeast granules (Lewis 1960). The stock has been maintained since 1970 in four large (several thousand flies), outbred population cages (Partridge & Farquhar 1983) of dimensions 30 × 15 cm and 20 cm high. Each population was fed with three bottles of food medium per week. These four populations were mixed into one single large population approximately 1 year prior to experiments to promote genetic variability in our experimental flies. Previous studies have also shown that this stock exhibits substantial levels of genetic variation (Wilkinson et al. 1990; Whitlock & Fowler 1996; Gardner et al. 2005), and experimental evolution studies show that this population contains selectable variation for a range of life history, behavioural and physiological traits (e.g. Sgrò et al. 1998, 2000; Sgrò & Partridge 1999; Wigby & Chapman 2004; Wigby et al. 2009). The Dahomey stock is maintained with overlapping generations to minimize selection on replication rate and life span. Therefore, related individuals can interact (and mate) in the Dahomey population both within and across generations.

Virgins were collected within 8 h of eclosion using ice anaesthesia. To obtain parents of the experimental flies, eggs were collected and raised at standard density (ca. 100 flies per bottle; Clancy & Kennington 2001). Virgin adults were placed in same-sex vials and aged for 1 week before single males and females were paired in vials to produce families. The parental pair was removed after 24 h and the eggs left to develop. To create individuals that were related and familiar to one another (R_f) siblings were raised from egg to adult together in the same vial. To generate individuals that were unrelated and unfamiliar to one another (U_u) nonsiblings were raised in separate vials. Virgin adults emerging from these vials were used for experimental trials.

To investigate the potential effects of social familiarity on inbreeding avoidance, we raised siblings in separate vials to create individuals that were related but unfamiliar (R_u). This emulates natural situations in which adult females lay eggs in spatially separated substrates. By comparing mating responses to R_f and to R_u individuals, we investigated the effects of familiarity while controlling for relatedness. Similarly, by comparing mating responses to R_u and to U_u flies, we examined the effects of relatedness while controlling for social familiarity. To create R_u individuals, the food medium containing unhatched eggs was split in half after the removal of the parental flies: half was transferred into a separate vial and all vials were supplemented with fresh medium. We standardized average egg density across treatments by discarding half of the medium in the R_f and U_u treatments and replacing it with fresh yeast medium. This procedure removed any potential familiarity effects caused by shared larval environment since none of the larvae hatched prior to separation of the eggs. All experiments were conducted blind with respect to relatedness and familiarity between individuals.

Inbreeding Depression

To investigate the cost of inbreeding, we quantified egg–adult viability of 1-day posteclosion (24–36 h posteclosion) females mated to either a same-aged R_f ($N = 40$) or U_u ($N = 40$) male. Males were removed and females allowed to oviposit in individual vials for 24 h postmating before they too were removed. We measured egg–adult viability as the ratio of eclosed adults to oviposited eggs in the vials 12 days after the oviposition period. Because the majority of the flies eclosed 10 days after oviposition, allowing 12 days before fly collection provided ample time for development.

Inbreeding Avoidance

Theory predicts that inbreeding avoidance is sex specific and changes with the availability of unrelated partners (e.g. Kokko & Ots

2006; Puurtinen 2011). To disentangle the roles of male and female behaviour in inbreeding avoidance, we used three types of assays: (1) an individual female was exposed to two males (female–male–male; FMM), (2) an individual male was exposed to two females (male–female–female; MFF), and (3) single females were placed with single males (male–female; MF). In the MF assay, effects of relatedness on the mating behaviour of the flies could be an outcome of female or male preference. In MFF and FMM, there is also a possibility for intrasexual interactions and an opportunity for the single male or female, respectively, to compare the two opposite-sex individuals and thus choose between them.

In the FMM and MFF assays, the two same-sex flies were marked on the thorax with either red or orange acrylic paint (Nilsen et al. 2004) in a randomized balanced design with respect to their relatedness to the fly of the opposite sex, to allow recognition. Males for FMM and MF assays as well as females for MFF were aspirated into individual vials 12–14 h prior to the trial. At lights-on the following morning, a single, same-aged individual of the opposite sex was aspirated into the vial, and mating behaviour was observed until mating (in the 'mate choice', 'social familiarity' and 'primary receptivity' experiments, see below) or remating (in the 'mating history' experiment, see below) had been observed in at least 85% of the vials. FMM, MFF and MF assays were used to answer specific questions in the different experiments outlined below. Assays within each experiment were conducted at the same time using the same batch of flies.

Mate choice

Using FMM and MFF assays, we examined whether focal individuals avoided inbreeding when given a choice of two opposite-sex individuals (Fig. 1a). Each of 45 focal females and 47 focal males were aspirated individually into vials with two virgin members of the opposite sex, of which one was R_f and the other U_u to the focal individual. All flies in this experiment were 1-day posteclosion virgins.

Social familiarity

To examine the effects of social familiarity on inbreeding avoidance, we conducted two sets of FMM assays, exposing females to two male types in the following combinations: (1) U_u and R_u ($N = 52$) and (2) R_f and R_u ($N = 50$). In addition, we included one set of MF observations of R_u pairs ($N = 45$), R_f pairs ($N = 45$) and U_u pairs ($N = 45$; Fig. 1b). All flies in these experiments were 1-day posteclosion virgins.

Primary receptivity

We examined the response of 1-day posteclosion (24–36 h posteclosion) virgin females ($N = 44$) and 2-day posteclosion (48–54 h posteclosion) virgin females ($N = 49$), each presented with two males, one R_f and one U_u, of similar time posteclosion as the focal female (Fig. 1c).

Mating history

To assay the effect of first mating on subsequent inbreeding avoidance, we initially mated females to either an R_f ($N = 41$) or a U_u ($N = 43$) male. Immediately following the first mating, females were placed with two novel males (FMM): an R_f and a U_u male (Fig. 1d). In treatments in which females were first mated to R_f males and thereafter presented with both a U_u and an R_f male, the second R_f male was the full sib brother of the first male. Therefore, to control for between-male relatedness, females that had first mated with a U_u male were presented subsequently with a new U_u male that was the full sib brother of the first U_u male.

Similarly, males were initially mated with an R_f female ($N = 42$) or a U_u female ($N = 44$) and thereafter presented with a choice of one R_f and one U_u female (Fig. 1d). The first and second U_u female used in a single trial were full siblings. All flies were 1-day post-eclosion at the time of the first mating.

We measured four aspects of sexual behaviour (Pekkala et al. 2009).

(1) Courtship behaviour before mating (orienting, tapping, wing vibration, licking and attempting copulation) in which we recorded the occurrence of courtship events in 1 min spot-checks until mating occurred. Male *Drosophila* must perform courtship before mating (Spieth 1952) and courtship of *D. melanogaster* males typically consists of periods of courting and not courting. The number of courtship counts directed at a particular female would be indicative of male preference whereas the amount of courtship required by the female to mate would reflect the female's receptivity to mate with either mate type. The number of spot-checks corresponded to the number of minutes between the start of the trial and the start of mating, which was measured as the latency to mating.

(2) Latency to mating. This is the time from the start of a trial to the start of mating. Latency to mating could be determined by female receptivity and male activity.

(3) Duration of mating. This is the time between the start and end of mating. Although traditionally thought to be mainly under male control (Wigby et al. 2009), mating duration can be modulated by female genotype (Goodwin et al. 2010).

(4) Type of partner that mated with the focal individual (i.e. R_f, U_u or R_u). This could be influenced by female choice and activity of the male.

Statistical Analysis

All statistical analyses were conducted using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, U.S.A.). To test for the effect of relatedness on egg–adult viability, we used a generalized linear model with binomial error distribution. Egg–adult viability was entered as the response variable and parental relatedness as the fixed factor. We also calculated the coefficient of inbreeding depression δ (Lande & Schemske 1985):

$$\delta = 1 - (X_I/X_O),$$

where X_I = inbred egg–adult viability and X_O = outbred egg–adult viability.

In the 'inbreeding avoidance' experiments, we used chi-square tests to test whether the focal individuals mated preferentially with either mate type. For FMM and MMF assays, we analysed variation in mating latency using analysis of covariance (ANCOVA). Mating latency was the dependent variable, and mate type (R_f, U_u or R_u) was added as the fixed factor. As a covariate, we included courtship counts (sum of frequencies of all different courtship behaviours) by the mated male relative to total number of courtship events by both males, or courtship counts directed towards the mated female relative to total courtship counts to both females. For the MF assay, we entered courtship counts in our ANCOVA model. Courtship counts were included as a covariate in the above analyses because courtship effort has a strong stimulating effect on females' receptivity (Kowalski et al. 2004). Thus, courtship effort was entered in the statistical models to account for some of the variance in mating latency. We used two-tailed *t* tests to compare mating duration with either mate type in FMM and MFF assays and we used ANOVA to compare mating duration between the three mate types in MF assays. Post hoc Tukey's tests were carried out to identify which of the three groups differed from each other. To achieve homogeneous variances and normality of residuals, we log transformed mating latency and mating duration data prior to analysis.

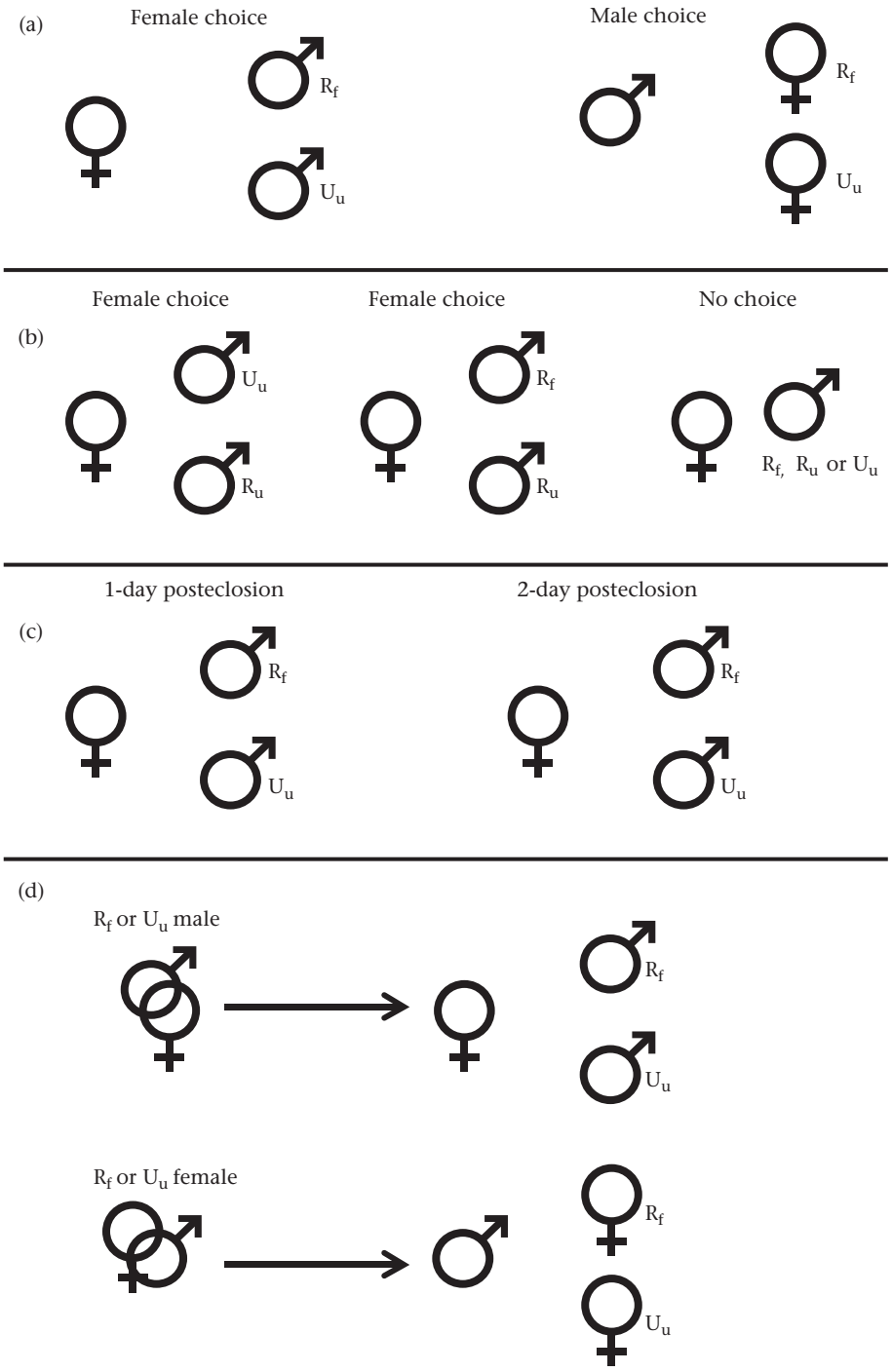

Figure 1. Experiments used to investigate inbreeding avoidance. R_f = related/familiar, U_u = unrelated/unfamiliar and R_u = related/unfamiliar. (a) 'Mate choice' experiments in which single females or males were introduced to two opposite-sex individuals. (b) 'Social familiarity' experiments in which single females were placed with two males or single males. (c) 'Primary receptivity' experiments in which either 1-day posteclosion or 2-day posteclosion virgin females were presented with both an R_f and a U_u male. (d) 'Mating history' experiments in which focal individuals were mated with either an R_f (related/familiar) or U_u (unrelated/unfamiliar) partner and subsequently presented with both an R_f and a U_u individual.

To investigate variation in female choice in the FMM assay, we compared the amount of courtship before mating with either the related or unrelated male, using *t* tests. Amount of courtship was the sum of counts of the various courtship behaviours. For the analysis of courtship effort in the MFF assays, we compared courtship counts directed by the male to the related versus unrelated female using paired *t* tests. We used a one-way ANOVA to compare the courtship effort of males from different treatment groups (R_f, R_u or U_u) in the MF assays. To account for multiple testing (four variables measured per assay), we used Bonferroni correction. Thus, *P* values were considered significant only when $P < 0.05/4 = 0.013$. In our study, the minimum sample size WAS 38. This means that we had a high (0.67) probability of detecting a large effect (Cohen's $d = 0.8$) and a relatively low probability (0.09) of detecting a small effect size (Cohen's $d = 0.2$).

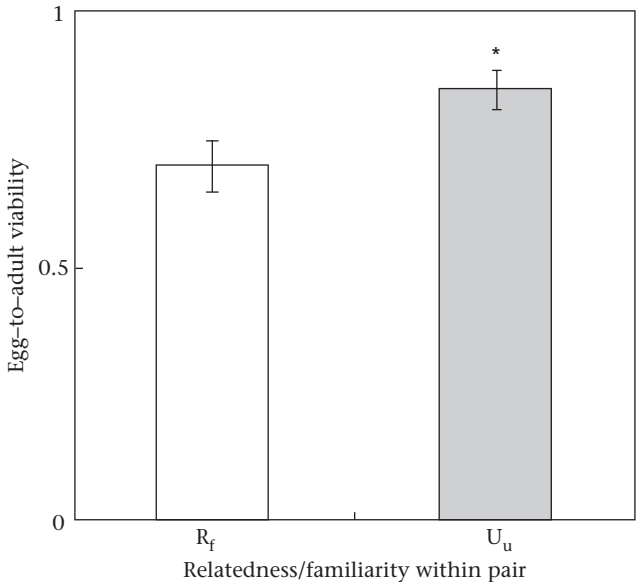

Figure 2. Egg–adult viability of offspring sired by R_f = related/familiar or U_u = unrelated/unfamiliar parents. Error bars denote SE. *$P < 0.05$.

RESULTS

Inbreeding Depression

There was no difference in the number of eggs laid by females mated to either an R_f (mean $\pm$ SE $= 21.75 \pm 0.96$) or a U_u male (21.30 ± 0.96; t test: $t_{78} = 0.33$, $P = 0.74$). Therefore, we did not adjust egg–adult viability for egg numbers. We observed significantly lower egg–adult viability of offspring sired by brothers (mean $\pm$ SE $= 0.70 \pm 0.05$) than by unrelated males (0.85 ± 0.03; generalized linear model: $Z = 7.01$, $N = 78$, $P < 0.001$). Consistent with inbreeding depression, the offspring of full sib mating suffered from on average 17.6% lower egg–adult viability than the offspring of unrelated parents (Fig. 2). The coefficient of inbreeding depression is therefore relatively low ($\delta = 0.176$).

Inbreeding Avoidance

Mate choice

There was no evidence of inbreeding avoidance in either the FMM or the MFF assays (Table 1 'Mate choice'). Males in the MFF assay did not preferentially court unrelated females and in the FMM, there was no difference in the amount of courtship required by the female to mate with either a related or an unrelated male (Table 1 'Mate choice').

Social familiarity

There was no evidence that social familiarity influenced responses to related and unrelated mates in the FMM assay (Table 1 'Social familiarity'). The amount of courtship required by females did not differ with male type (R_u, R_f or U_u). In the MF assay, latency to mating, probability of mating and courtship frequency did not differ between the three groups (Table 1 'Social familiarity'); R_u individuals mated for longer than R_f individuals (Tukey's test: $P = 0.015$), but at a marginally nonsignificant level after Bonferroni correction (Table 1 'Social familiarity').

Primary receptivity

As expected, latency to mating was significantly shorter for 2-day posteclosion virgins than for 1-day posteclosion virgins (1 day: 92.10 ± 7.55 min; 2 days: 15.45 ± 7.16 min; $F_{1,91} = 88.70$, $P < 0.001$), indicative of the difference in primary receptivity. However, we detected no evidence of inbreeding avoidance in either 1-day posteclosion or 2-day posteclosion virgin females of the FMM assays (Table 1 'Primary receptivity').

Mating history

Females first mated to U_u or R_f males also did not show a difference in amount of courtship required, remating probability, latency and duration with either male type (Table 1 'Mating history'). Females that had first mated to R_f males showed a nonsignificant trend towards mating faster with U_u males (Table 1 'Mating history'). In the MFF assays, males that were first mated to U_u females were slower to remate with U_u females then they were to remate with R_f females (Table 1 'Mating history'; Fig. 3). However, remating duration, probability of remating with R_f or U_u females and proportion of courtship directed to each female type did not differ (Table 1 'Mating history'). Males first mated to R_f females showed no difference in the latency to remating, duration of remating and proportion of courtship to either mate type (Table 1 'Mating history').

DISCUSSION

We found significant but low inbreeding depression and little evidence of precopulatory inbreeding avoidance, after controlling for sex-specific behaviours, social familiarity, primary receptivity and mating history. Our results contrast with some previous studies showing inbreeding avoidance in invertebrates using virgin individuals (e.g. Simmons 1991; Lihoreau et al. 2008) and, in particular, in *D. melanogaster* (Averhoff & Richardson 1974, 1976; Tompkins & Hall 1984; but see van den Berg et al. 1984). It is worth noting that previous studies on *D. melanogaster* imposed pair mating between full siblings for several generations. This may result in selection for highly inbred flies that exhibit mating behaviour not representative of less inbred populations (Miller et al. 1993; Miller & Hedrick 1993, 2001). The different inbreeding protocols used could have resulted in directional selection for or against inbreeding avoidance mechanisms. The preference for unrelated partners in Averhoff & Richardson's (1974, 1976) studies could be a consequence of the importance of restoring female fitness when inbreeding depression is substantially high. Conversely, the inbreeding regime used by van den Berg et al. (1984) might have purged deleterious alleles in the population, eliminating the need for inbreeding avoidance. Flies used in our study were not inbred prior to experimentation and thus might not show inbreeding avoidance because the cost of inbreeding is relatively low compared to that of inbred lines. Theory predicts that for low or no male parental investment, both males and females should mate with a full sibling if the coefficient of inbreeding depression is lower than 1/3 (Parker 1979, 2006). The coefficient of inbreeding depression calculated from egg–adult viability in our study was 0.176. While this corresponds to a relatively large amount of inbreeding depression (DeRose & Roff 1999), it would appear not to be sufficiently high to generate strong selection for inbreeding avoidance. Therefore, the lack of inbreeding avoidance mechanisms observed in this study appears broadly consistent with theoretical predictions, particularly in light of limited male parental investment in this species. However, our study did not quantify the effects of inbreeding on adult survival and reproductive success, and thus might have underestimated inbreeding depression somewhat. Nevertheless, the proportion of inbreeding depression missed by our study would have to be substantial to very large (relative to inbreeding depression for life history traits, DeRose & Roff 1999) for inbreeding avoidance to evolve (i.e. at least 0.16 for inbreeding avoidance to be selected in

Table 1
Mating responses of *D. melanogaster* in inbreeding avoidance experiments

Response variable	Factors	Values (mean±SE)	*df*	Test statistic	*P*
Mate choice					
FMM					
Courtship counts	Relatedness	R_f: 2.09±0.49; U_u: 2.14±0.50	42	0.097	0.923
Mating latency	Relatedness	R_f: 31.58±10.88; U_u: 40.52±8.35	41	1.200	0.280
Mating duration	Relatedness	R_f: 23.95±1.52; U_u: 20.96±1.04	42	0.529	0.102
Mating probability	Relatedness	R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.818	0.366
MFF					
Courtship counts	Relatedness	R_f: 2.19±0.21; U_u: 1.83±0.20	45	1.258	0.799
Mating latency	Relatedness	R_f: 36.54±4.83; U_u: 34.57±6.00	44	0.288	0.739
Mating duration	Relatedness	R_f: 22.81±0.64; U_u: 23.95±1.09	45	0.416	0.679
Mating probability	Relatedness	R_f: 0.55±0.07; U_u: 0.45±0.07	1	0.532	0.466
Social familiarity					
FMM					
Courtship counts	U_u vs R_u	U_u: 2.49±0.95; R_u: 1.43±0.41	48	1.153	0.255
	R_f vs R_u	R_f: 2.21±0.53; R_u: 2.21±0.80	46	<0.001	1.000
Mating latency	U_u vs R_u	U_u: 40.23±8.80; R_u: 29.44±8.62	47	0.145	0.705
	R_f vs R_u	R_f: 32.95±6.76; R_u: 22.61±7.86	45	0.343	0.561
Mating duration	U_u vs R_u	U_u: 15.96±1.27; R_u: 16.50±1.20	48	0.055	0.954
	R_f vs R_u	R_f: 16.96±1.30; R_u: 19.50±1.48	46	1.012	0.932
Mating probability	U_u vs R_u	U_u: 0.49±0.07; R_u: 0.51±0.07	1	0.020	0.889
	R_f vs R_u	R_f: 0.57±0.07; R_u: 0.43±0.07	1	1.042	0.307
MF					
Courtship counts	Relatedness/familiarity	R_f: 3.95±0.62; U_u: 4.66±0.77; R_u: 3.03±0.65	103	1.407	0.250
Mating latency	Relatedness/familiarity	R_f: 49.70±6.73; U_u: 47.82±8.13; R_u: 64.69±9.24	102	1.140	0.324
Mating duration	Relatedness/familiarity	R_f: 16.32±0.87; U_u: 15.48±0.84; R_u: 18.97±1.06	103	4.459	0.014
Mating probability	Relatedness/familiarity	R_f: 0.82±0.06; U_u: 0.67±0.07; R_u: 0.77±0.06	2	3.093	0.213
Primary receptivity					
FMM					
Courtship counts	Relatedness (1-day posteclosion)	R_f: 3.80±0.76; U_u: 3.70±9.82	43	0.251	0.803
	Relatedness (2-day posteclosion)	R_f: 2.37±0.53; U_u: 2.73±0.61	48	0.701	0.487
Mating latency	Relatedness (1-day posteclosion)	R_f: 89.47±11.48; U_u: 94.73±9.82	42	0.014	0.908
	Relatedness (2-day posteclosion)	R_f: 19.78±10.44; U_u: 11.12±9.82	47	3.628	0.063
Mating duration	Relatedness (1-day posteclosion)	R_f: 17.90±0.89; U_u: 18.04±0.76	43	0.071	0.908
	Relatedness (2-day posteclosion)	R_f: 17.52±0.80; U_u: 17.19±0.76	48	0.327	0.745
Mating probability	Relatedness (1-day posteclosion)	R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.083	0.943
	Relatedness (2-day posteclosion)	R_f: 0.47±0.07; U_u: 0.53±0.07	1	0.818	0.366
Mating history					
FMM					
Courtship counts	First mating R_f	Second mating R_f: 6.36±0.73; U_u: 6.41±1.10	38	0.048	0.962
	First mating U_u	Second mating R_f: 6.12±0.67; U_u: 5.12±0.65	40	1.533	0.133
Remating latency	First mating R_f	Second mating R_f: 351.4±42.64; U_u: 250.1±42.64	37	4.471	0.041
	First mating U_u	Second mating R_f: 286.48±37.35; U_u: 289.16±41.09	39	0.010	0.919
Remating duration	First mating R_f	Second mating R_f: 17.75±1.37; U_u: 15.65±1.37	38	0.897	0.286
	First mating U_u	Second mating R_f: 16.13±1.34; U_u: 16.16±1.47	40	0.014	0.989
Remating probability	First mating R_f	Second mating R_f: 0.50±0.08; U_u: 0.50±0.08	1	0.000	1.000
	First mating U_u	Second mating R_f: 0.55±0.08; U_u: 0.45±0.08	1	0.381	0.537
MFF					
Courtship counts	First mating R_f	Second mating R_f: 2,14±0.20; U_u: 1.86±0.20	40	0.543	0.590
	First mating U_u	Second mating R_f: 2.50±0.28; U_u: 3.50±0.43	42	1.504	0.140
Remating latency	First mating R_f	Second mating R_f: 49.33±15.45; U_u: 39.86±15.45	39	0.916	0.344
	First mating U_u	Second mating R_f: 55.37±26.52; U_u: 130.20±23.12	41	6.870	**0.012**
Remating duration	First mating R_f	Second mating R_f: 18.62±1.44; U_u: 18.48±1.44	40	0.228	0.821
	First mating U_u	Second mating R_f: 18.68±1.21; U_u: 18.52±1.05	42	0.520	0.606

Table 1 (*continued*)

Response variable	Factors	Values (mean±SE)	*df*	Test statistic	*P*
Remating probability	First mating R_f	Second mating R_f: 0.50±0.08; U_u: 0.50±0.08	1	0.000	1.000
	First mating U_u	Second mating R_f: 0.43±0.08; U_u: 0.57±0.08	1	0.818	0.366

R_f = related/familiar, U_u = unrelated/unfamiliar and R_u = related/unfamiliar partners. 1-day posteclosion, $N = 24–36$ h posteclosion and 2-day posteclosion, $N = 48–54$ h posteclosion. Values for mating latency and mating duration are in minutes and nontransformed values are presented for the purpose of visualization. Values for mate choice represent the mating probability. Test statistic values were based on the *t* distribution except for 'mating/remating probability' where the test statistic is based on the chi-square distribution and 'mating latency' where the test statistic is based on the *F* distribution. *P* values that are significant after sequential Bonferroni correction ($P < 0.013$) are highlighted in bold.

females, and at least 0.50 for inbreeding avoidance to be selected in males). It is difficult to estimate the magnitude of inbreeding depression arising after offspring reach adulthood because few studies have attempted to quantify this component of inbreeding depression, and because the amount of inbreeding depression is strongly contingent on the breeding regimes, population parameters and environmental conditions of different populations (Sharp 1984; Mackay 1985). However, there is some evidence suggesting that inbreeding depression arising from adult reproductive success might be modest for one generation of inbreeding. For example, Tantawy & Reeve (1956) found no reduction in net fertility following one generation of sib–sib mating (but substantial inbreeding depression in fertility following successive generations of inbreeding). Similarly, Hughes (1996) found no inbreeding depression in male fertility. Swindell & Bouzat (2006) measured inbreeding depression in lineages of *D. melanogaster* maintained under different levels of ancestral inbreeding, as the 72 h production of individual females, a measure that takes into consideration both egg–adult survival and some reproductive success. Mean inbreeding depression ranged from 0.27 to 0.09 across different ancestral inbreeding treatments. In addition to demonstrating the variability of inbreeding depression, these results show that even when a more inclusive measure of offspring fitness is used, inbreeding depression in *D. melanogaster* is unlikely to exceed the 0.33 threshold. Inbreeding depression caused by reproductive performance alone was also low in a population of prairie voles, *Microtus ochrogaster* (Bixler & Tang-Martinez 2006), indicating that this pattern is more broadly plausible.

The use of the Dahomey population in our study provides advantages and potential caveats. An advantage is that flies were assayed in an environment to which they are adapted (since 1970) and is thus likely to represent behaviour that occurs in the laboratory cages. However, the behaviour may not be representative of wild populations of *D. melanogaster*. It is possible that the maintenance of large, well-mixed, population cages might have relaxed selection on inbreeding avoidance mechanisms compared to the wild. For example, the cage environment may reduce the probability of mating with a relative owing to the large number of flies within close proximity. Using freshly extracted flies from the field would be more reflective of wild populations. However, this approach would also not be without caveat. First, wild flies might not behave naturally when placed in the laboratory, a novel environment to which they are not adapted. Second, adaptation to the laboratory is rapid in *D. melanogaster* (e.g. Frankham & Loebel 1992) and thus recently caught flies are likely to be assayed while undergoing a process of intense selection. Males of the Dahomey stock have recently been shown to exhibit strategic copulation behaviour in response to the presence or absence of rival males (Wigby et al. 2009; Bretman et al. 2009, 2010): a behaviour that would have evolved in the wild and would be predicted to undergo relaxed selection in the laboratory where numerous rivals are always present. Thus, there is reason to expect that if inbreeding avoidance behaviour was present in the wild ancestral population, we would be able to detect it in our present study. No one approach is ideal, but the respective limitations of using recently caught versus laboratory-adapted populations should be considered when drawing conclusions.

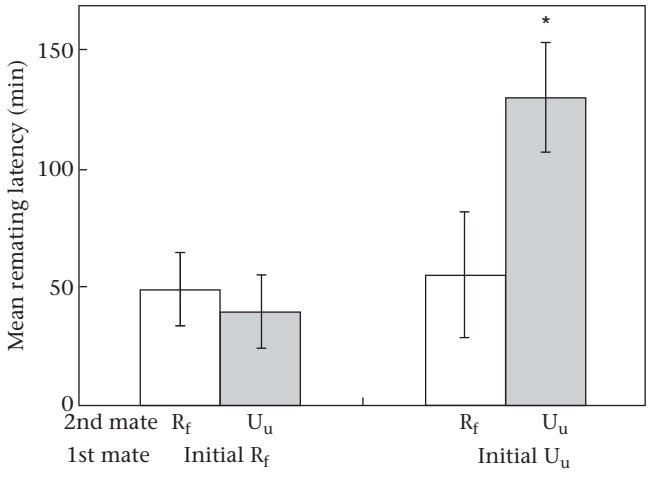

Figure 3. Remating latency in the 'mating history male choice' experiment (Fig. 1). R_f = related/familiar, U_u = unrelated/unfamiliar. Error bars denote SE. *P values that are significant after sequential Bonferroni correction ($P < 0.013$).

In the FMM and MFF assays, although focal individuals were presented with a choice, it was impossible to rule out potential effects of intrasexual competition. We observed no female–female interactions during our MFF trials, and so it is unlikely that such interactions confound the results of these assays. Observations of contact between the males in most of the FMM mating trials and reduced courting of the males in the presence of another male indicates interaction between the males (Table 1). Male–male interactions could potentially mask female inbreeding avoidance if the related male was particularly successful in competing with the unrelated male or that the former was relatively more intense in courting the female, for example, if one male was larger or in better condition than the other. However, all males were reared under identical conditions and thus variation in body size and condition was minimized and random across treatments. Moreover, it is unlikely that related males courted females more intensely because we detected no difference in the amount of courtship required by the female to mate with either the related or unrelated male. Therefore, although we cannot unequivocally exclude male–male interactions as a potential explanation for the results in our FMM and MMF assays, all the available evidence suggests that they were unlikely to mask female inbreeding avoidance.

In contrast to a previous study demonstrating female's preference to mate with unfamiliar males (Ödeen & Moray 2008), we detected no effect of social familiarity on female mate choice in our

'Social familiarity' experiment. Females were equally likely to mate with related/familiar males and with related/unfamiliar males. This is probably due to the difference in designs between our study and that of Ödeen & Moray (2008). We did not house females with males behind a piece of netting to allow for 'familiarization' prior to trials. This treatment might have been necessary for females to distinguish between previously encountered and not previously encountered males.

Most of our experiments involved virgin flies. Virgin *D. melanogaster* of both sexes are typically eager to mate, which could potentially mask important behaviours that are present only in previously mated individuals. Given that, like most animals, male and female *D. melanogaster* typically mate multiply (Harshman & Clark 1998; Imhof et al. 1998) the mating behaviour of nonvirgin individuals needs to be addressed in more studies. In our experiments we found that males first mated to unrelated females took significantly longer to remate with another unrelated female than with a novel related female, suggesting that mating history could potentially play an important role in mediating inbreeding likelihood.

Despite little evidence of precopulatory inbreeding avoidance in our study, it is possible that other mechanisms exist in this species to minimize mating with kin. First, polyandry might reduce the number of inbred offspring produced by a female where a brood is sired by multiple males (Harshman & Clark 1998; Imhof et al. 1998; Michalczyk et al. 2011; but see Hosken & Blanckenhorn 1999). Second, postcopulatory inbreeding avoidance might occur in *D. melanogaster*, where sperm competitive ability is shown to be negatively correlated with relatedness (Mack et al. 2002; Panhuis & Nunney 2007). However, a recent experimental test found no evidence of this (Ala-Honkola et al. 2011). Moreover, Ala-Honkola et al. (2011) also found no evidence of precopulatory inbreeding avoidance in no-choice experiments between related and familiar virgin flies, which is consistent with the findings of our study. In conclusion, our results suggest that, although there was significant inbreeding depression, precopulatory inbreeding avoidance is absent in our study population. This is not surprising as the magnitude of inbreeding depression in our population is less than the threshold estimated by theoretical models.

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