



## Preference for related mates in the fruit fly, *Drosophila melanogaster*

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Although inbreeding is known for its negative consequences, recent theory predicts that inclusive fitness gains from inbreeding should outweigh the costs of inbreeding depression in many situations, resulting in optimal fitness from mating with intermediate relatives (optimal inbreeding). Consistent with this theory, in a previous field study on *Drosophila melanogaster*, we found that males were more closely related to their mate than to females sampled randomly from the same population. However, in that study relatedness was higher than expected between all individuals collected mating, not just actual pairs, suggesting factors other than mate choice may have been at play. In the present study we aimed to gain a more detailed understanding of how relatedness influences male and female mate preference in *D. melanogaster* by investigating preference between pairs of known relatedness, under controlled conditions. We used multivariate techniques to investigate linear and nonlinear effects of relatedness while accounting for variation in morphological traits thought to be under sexual selection. Consistent with the field results, we found that females accepted courtship from closely related males more rapidly. We detected no influence of relatedness on the largely male controlled traits, latency to first courtship and copulation duration. These results are consistent with optimal inbreeding theory and provide weight to the idea that there may need to be a shift in the way we think about inbreeding. More work is warranted in this area.

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Inbreeding is best known for its severe negative consequences (reviewed in Charlesworth & Willis 2009). However, it has been argued that despite the costs of inbreeding depression, individuals could choose to mate with related individuals because of inclusive fitness gains (Parker 1979). More recently Kokko & Ots (2006) demonstrated theoretically that, under a wide range of natural situations and realistic values of inbreeding depression, inclusive fitness gains can favour remarkably high levels of inbreeding tolerance. Lehmann & Perrin (2003) suggested that a balance between inbreeding depression and inclusive fitness benefits may result in an intermediate level of inbreeding providing optimal fitness (a concept known as optimal inbreeding). Puurtinen (2011) took the concept of inclusive fitness benefits further and, through modelling, derived the stable level of inbreeding expected in populations practising mate choice based on relatedness for optimal inclusive fitness. This theory is supported by empirical studies of a number of species, demonstrating either mate preference for, or biased paternity towards, close or intermediate relatives under controlled laboratory conditions (Bateson 1982; Barnard & Fitzsimons 1988, 1989; Keane 1990; Schjorring & Jager 2007;

Thunken et al. 2007; Richard et al. 2009) and in natural populations (e.g. Krokene & Liffeld 2000; Cohen & Derhorn 2004; Kleven et al. 2005; Sherman et al. 2008; Wang & Lu 2011).

In a previous study of a large field population of the fruit fly *Drosophila melanogaster*, we found evidence suggesting that this population may have been optimally inbreeding (Robinson et al. 2009). Specifically, we found clear evidence that individuals were not mating randomly with respect to relatedness; the average relatedness ( $R \pm SE$ ) between pairs collected mating in the field was  $0.16 \pm 0.014$  (Robinson et al. 2012). Males, but not females, were more closely related to their mate than to a randomly sampled opposite-sex individual from the population. These results suggest that there may be mate preference for inbreeding in this population. However, we also found that all mating individuals were more closely related to each other than were randomly sampled individuals ( $R = 0.012 \pm 0.005$ ). While the average level of relatedness calculated between pairs in the field gives some indication of the outcome of mating events, this outcome may arise either because of active mate choice based on level of relatedness or, for some reason, because of the level of relatedness of individuals actually available to mate (Robinson et al. 2012). Our goal in the present study was to increase our understanding of mate preference for relatedness in both males and females of this population of *D. melanogaster* using an experimental approach. We conducted mate choice trials in which the time to courtship, interval between courtship and copulation,

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and copulation duration could be measured between isolated pairs of individuals of known levels of relatedness. This approach avoids the possible complications of male–male or female–female interactions associated with simultaneous choice experiments.

Previous studies investigating the effects of relatedness on mate choice have usually considered relatedness in isolation (e.g. Bateson 1982; Keane 1990; Cohen & Derhorn 2004; Frommen & Bakker 2006). However, as Blows (2007) pointed out, selection does not act on traits in isolation and analysing components of fitness individually may misrepresent the form of selection that is acting upon them. Therefore, as we did in our field-based study (Robinson et al. 2012), we used a multivariate approach to consider how selection acting on relatedness interacts with selection on wing size and sex comb size, two traits thought to be under sexual selection in this species (Taylor & Kekic 1988; Ahuja & Singh 2008). Because theory predicts that an intermediate level of inbreeding may be optimal (Puurtinen 2011), we also used response surface methodology and canonical analysis to test for nonlinear effects.

## METHODS

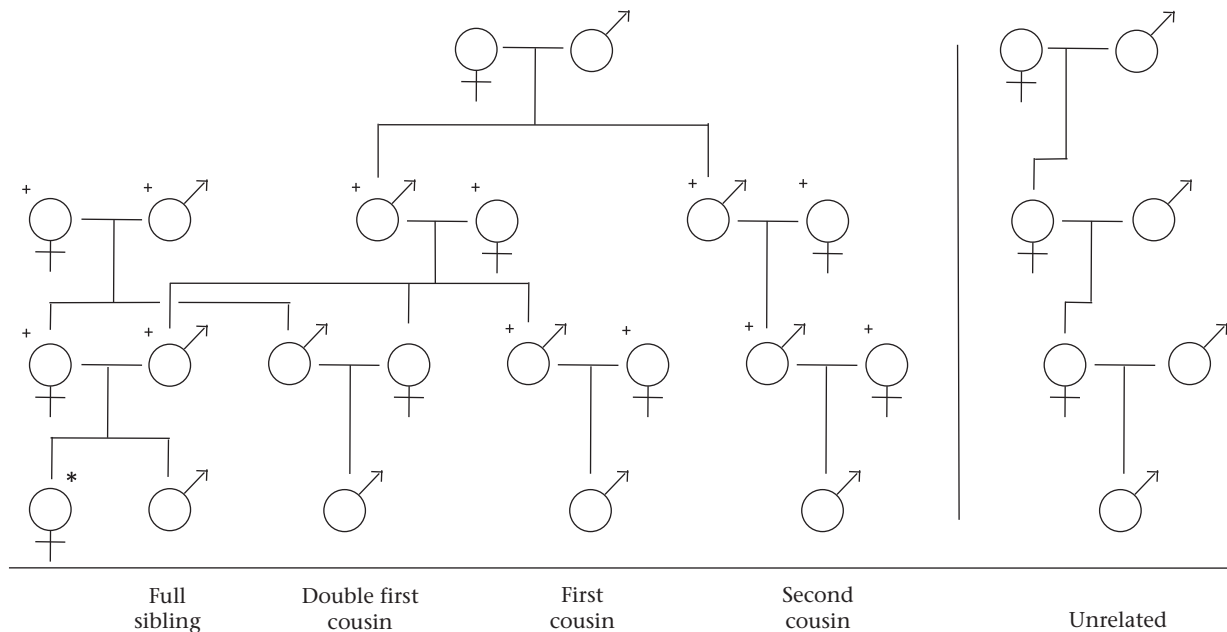
### Generation of Experimental Flies

The *D. melanogaster* stock used in this experiment was established in May 2009 from 151 wild-caught females collected from our field site in Margaret River (located on the southwest coast of Western Australia, 33.95°S, 115.07°E; Robinson et al. 2012). All fly stocks and experimental flies were maintained on standard agar–maize–yeast medium at 25 °C with a 12:12 h light:dark cycle. Stocks were maintained en masse in 9-litre containers with overlapping generations. Pedigree lines were generated approximately 1 year after the stock population was established. We used a crossing design, which, after four generations, generated focal females from a single family with individuals of known relatedness to them: full siblings ( $R = 1/2$ ), double first cousins ( $R = 1/4$ ), first cousins ( $R = 1/8$ ), second cousins ( $R = 1/32$ ) and unrelated individuals ( $R \approx 0$ ; Fig. 1). We generated 20 replicate blocks, 10 with

males used to establish each subsequent cross and 10 using females to establish each subsequent cross (see Fig. 1). Ultimately, within each of the 10 pedigrees we generated five replicates of each of the five levels of relatedness, giving a sample size of 500 pairs for the mate preference assay. To generate the pedigrees, flies were paired in individual 50 ml vials that contained 10 ml of food medium and that had been seeded with approximately 5 mg of live yeast. To ensure larval density remained low, adults were transferred to a second laying vial after 48 h and then discarded after a further 48 h. Individuals from these two vials were collected after 14 days using brief (less than 60 s) CO<sub>2</sub> anaesthesia, and paired with virgins from the stock population to establish the next set of crosses. All virgins used throughout the experiment were collected under cold anaesthesia and to ensure they were collected before reaching sexual maturity, they were sorted into single-sex vials within 8 h of emerging (Goldstein & Fyrberg 1994). As an additional step each single-sex vial was kept for 3 days prior to use, to confirm no offspring were produced in this time. To ensure the pedigree was complete, even if some crosses produced no offspring, four replicates of each cross were established and one of these was selected at random to provide offspring for the next step in the pedigree.

### Measures of Mate Preference

In *Drosophila*, courtship consists of a complex series of ordered behaviours performed by the male. These include orienting, following, tapping, singing (wing extension and vibration) and licking, which convey visual, olfactory, gustatory, auditory and tactile cues, allowing males and females to recognize and evaluate potential mates (Pan et al. 2011). Cues gained during courtship and copulation may allow individuals to assess how closely related potential mates are and adjust their response accordingly. In particular, chemical cues, such as cuticular hydrocarbons (CHCs), known to be important in sexual signalling in both male and female *D. melanogaster* (Jallon 1984), may convey information on relatedness as they are thought to be involved in species, sex and kin recognition in insects (Singer 1998).



**Figure 1.** Crossing design used to generate the desired levels of relatedness. The female marked with an asterisk indicates the focal female group, with which males of the desired levels of relatedness could be paired. Two separate pedigree designs were used. This figure shows the design where males were collected from the pedigree to establish subsequent crosses. For the pedigrees where females were used to establish each subsequent cross, the sex of all individuals marked '+' was the opposite of that shown here.

We assessed three important components of mating success that are indicators of mate preference: latency to courtship, interval between courtship and copulation, and copulation duration. The total latency to copulation has been commonly used as a measure of female preference in *Drosophila* (e.g. Pitnick 1991; Taylor et al. 2010). To get a more detailed picture of the mechanisms of nonrandom mating, we divided this into two components: latency to courtship and the interval between the commencement of courtship and the beginning of copulation. Initiation of courtship in *D. melanogaster* is thought to be primarily dependent on the male (Hall 1994) and is initiated in response to appropriate olfactory and visual cues (Greenspan 1995; Ejima & Griffith 2008). The commencement of copulation, on the other hand, is under female control and males of most species of *Drosophila* do not attempt to copulate unless the female gives an acceptance response (Spieth 1974).

Our third measure of mate preference, copulation duration, is an important component of male mating success in many insects and is often correlated with sperm transfer (Simmons 2001). While not correlated with sperm transfer in *D. melanogaster* (Lupold et al. 2011), increased copulation duration has been found to facilitate greater transfer of accessory gland proteins, leading to a longer refractory period in females and making it an important factor in male reproductive success (Gilchrist & Partridge 2000). In *D. melanogaster* copulation duration is considered to be largely under male control (Macbean & Parsons 1967), and recent studies suggest it is an important indicator of cryptic male mate choice (Bretman et al. 2009, 2011). None the less, work on the role females play in determining copulation duration suggests that the length of time a pair stays in copula cannot be considered to be entirely a property of the male (Markow 1996).

#### Mate Preference Assay

Approximately 17 h prior to the commencement of the assay, individual 4–7-day-old males of the desired levels of relatedness to the focal females were transferred to individual 50 ml vials containing food medium, using brief (less than 60 s) CO<sub>2</sub> anaesthesia. Transferring the males ahead of time allowed the males both to recover from the effects of the CO<sub>2</sub> and to explore their environment well before the assay. At the commencement of the assay, single 4–6-day-old females from the focal group for that block were aspirated into each vial using a mouth pooter; females were not anaesthetized. Pairs were allowed to interact for 80 min, by which time over 95% had copulated. The latency between the female being introduced into the vial and the male initiating courtship was recorded. Courtship was defined as when the male either pursued the female or displayed to her by flicking one of his wings out. If copulation occurred, the interval between the first courtship by the male and the commencement of copulation was recorded along with copulation duration. If a male was not observed to court a female, the pair was scored as taking the maximum time for courtship and excluded from the analyses of the interval between courtship and copulation and copulation duration. If a male courted, but did not copulate with a female, the pair was scored as taking the maximum time remaining for the interval between courtship and copulation and excluded from the analysis of copulation duration.

#### Morphological Traits

##### Wing size

In *D. melanogaster* wing size is strongly correlated with body size and is often used as a proxy for body size as wings can be measured more easily and accurately than other measures such as

thorax size (e.g. Gilchrist & Partridge 1999). We were interested in accounting for body size as it has previously been found to be under directional sexual selection in *D. melanogaster*, with larger males being more successful in obtaining mates (Taylor & Kekic 1988). Both male and female body size have also been found to influence copulation duration (Lefranc & Bundgaard 2000). The right wing area of each fly was measured using methods outlined in Robinson et al. (2012). In rare cases where the right wing was missing or damaged the left wing was measured in its place.

##### Sex combs

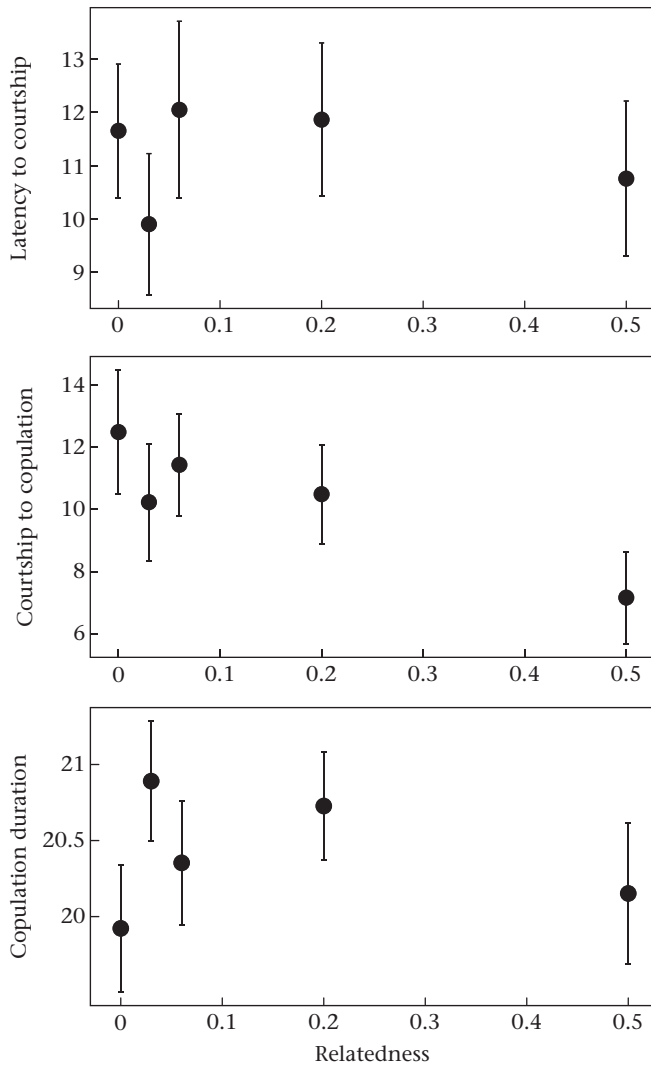
In *Drosophila*, the sex comb is a structure consisting of a row of modified bristles or 'teeth' located on the anterior ventral surface of the first pair of legs in males, and it is highly variable across species (Kopp & True 2002). The sex comb is thought to be involved in manipulating the female during copulation, and there is some evidence to suggest that the number of teeth making up the sex comb in *D. melanogaster* may be under sexual selection (Ahuja & Singh 2008). Sex comb tooth number has been found to affect mating success in the related species *D. simulans* and *D. bipunctata* (Markow et al. 1996; Polak et al. 2004). The number of teeth on the sex comb on each male's right leg was counted and used as a measure of sex comb size. This was achieved by removing the right foreleg, mounting it on a slide with double-sided tape and observing the sex comb under a Leica DMLS compound microscope at 200× magnification. Where the right leg was missing or damaged the left leg was measured in its place.

##### Statistical Analysis

Statistical analysis was carried out using R (GUI 1.40-devel Leopard build; R Development Core Team 2010) unless otherwise stated, and means are presented  $\pm 1$  SE. We used multiple linear regression and response surface methodology (Phillips & Arnold 1989; Blows & Brooks 2003) to investigate the linear and nonlinear effects of relatedness on courtship and copulation characteristics. These methods are widely used to estimate selection coefficients operating on phenotypic traits (e.g. Brooks et al. 2005). The linear component of these methods has also been used to investigate the effects of traits such as relatedness, which is a property of a pair of individuals (e.g. Wang & Lu 2011). Canonical analysis is particularly useful because it determines the combination of traits on which the greatest nonlinear effects are acting. This method is more sensitive to nonlinear effects such as those predicted by optimal inbreeding theory than other commonly used techniques such as second-order polynomial regression (Blows & Brooks 2003).

Before analysis the effect of block on dependent variables was factored out by dividing each score by the mean for that block. Where appropriate, to improve normality, dependent variables were transformed using log transformations, Yeo–Johnson power transformations (Yeo & Johnson 2000) or a combination of the two. After fitting linear models, we checked residual and normal QQ plots to confirm normality and adequacy of fit. The effect of block on explanatory variables was factored out by taking each score, subtracting the mean, and dividing by the standard deviation within each block. Explanatory variables were then converted to *z* scores with a mean of zero and standard deviation of one.

We constructed separate models to examine latency to first courtship, the interval between first courtship and copulation, and copulation duration. The explanatory variables were relatedness, wing size and sex comb tooth number (males only). Before undertaking each analysis, we excluded individuals with missing data for one or more traits. Linear relationships were tested using multiple linear regression including only main effects in the model.



**Figure 2.** Variation (min) of the three aspects of mate preference with relatedness. Data are presented as uncorrected means  $\pm$  SEs.

Nonlinear effects were investigated by fitting a response surface using the *rsm* function (*rsm* package version 1.40; Lenth 2009). The response surface was used to derive quadratic and correlational (interaction) coefficients as well as to calculate eigenvalues and canonical weights. Eigenvalues and quadratic regression coefficients were doubled as required when estimating nonlinear selection gradients (Stinchcombe et al. 2008). Significance testing of eigenvalues was conducted using two methods: first the classical double regression method (Phillips & Arnold 1989) and second, because of the high false positive rates associated with the double regression technique, the new permutation method recommended

**Table 2**

Eigen-analysis for nonlinear effects of predictor variables on latency to first courtship

	m1	m2	m3	m4
Eigenvalue	0.118	0.034	-0.006	-0.041
Double regression <i>P</i>	<b>0.003</b>	0.322	0.964	0.163
Permutation <i>P</i>	<b>0.017</b>	0.335	0.896	0.559
Canonical weights				
Relatedness	0.309	-0.352	0.787	0.401
Female wing area	-0.623	-0.120	0.494	-0.594
Sex comb	-0.499	-0.700	-0.320	0.398
Male wing	-0.517	0.609	0.184	0.572

Significant results are shown in bold ( $P < 0.05$ ).

by Reynolds et al. (2010) was used. Double regression was carried out by fitting a second-order polynomial linear model to the new multivariate variables. Permutation was carried out in R using code given in the supplementary material to Reynolds et al. (2010).

## RESULTS

### Latency to First Courtship

Raw data for the effect of relatedness on the three aspects of mate choice are presented in Fig. 2. Linear, correlational and quadratic effects for latency to first courtship are given in Table 1. There was a significant interaction effect between female wing area and male wing area, suggesting size-assortative mating for this trait. None of the other effects were statistically significant. The canonical analysis revealed one significant nonlinear axis of variation, the new multivariate variable m1 (Table 2). The eigenvalues associated with this new multivariate variable indicate that it is largely influenced by female wing area, male wing area and sex comb size. Fitting a cubic spline to this new variable reveals lowest latency to courtship at intermediate levels of m1 (Fig. 3). This indicates that combinations of pairs either with intermediate phenotypic characteristics or with relatively large individuals paired with relatively small individuals have the shortest latency to courtship times.

Of the 475 pairs observed, five males showed no sign of courting the female during the observation period. These pairs comprised one full-sibling pair, one double first-cousin pair, two first-cousin pairs and one second-cousin pair.

### Interval Between Courtship and Copulation

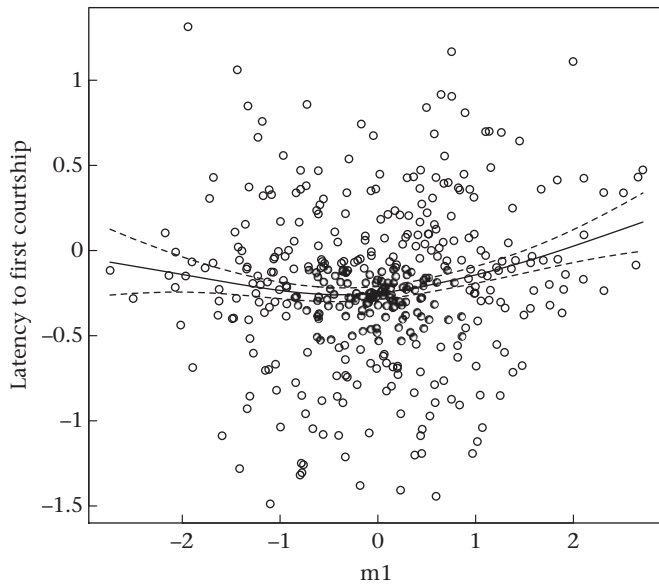
Coefficients for the linear, correlational and quadratic effects on interval between courtship and copulation are given in Table 3. There was a significant negative linear coefficient for the effect of relatedness, indicating that increased relatedness between pairs was associated with lower courtship to copulation intervals. There was also a significant linear effect of male wing area but in the opposite direction, with larger male wings being associated with

**Table 1**

The four linear coefficients and matrix of four quadratic and six correlational nonlinear coefficients for latency to first courtship  $\pm$  SEs

	Linear ( $\beta$ )	Nonlinear ( $\gamma$ )			
		Relatedness	Female wing area	Sex comb	Male wing area
Relatedness	-0.001 $\pm$ 0.024	0.003 $\pm$ 0.030	-0.014 $\pm$ 0.024	-0.015 $\pm$ 0.023	-0.036 $\pm$ 0.027
Female wing area	-0.038 $\pm$ 0.023		0.015 $\pm$ 0.016	0.050 $\pm$ 0.026	<b>0.049<math>\pm</math>0.020</b>
Sex comb	-0.031 $\pm$ 0.024			0.019 $\pm$ 0.020	0.007 $\pm$ 0.027
Male wing area	-0.012 $\pm$ 0.023				0.015 $\pm$ 0.015

Significant result is shown in bold ( $P < 0.05$ ).



**Figure 3.** Cubic smoothing spline fitted to the new multivariate variable  $m1$  and latency to first courtship. Open circles represent individual data points, the solid line represents the fitted spline and dashed lines represent 95% pointwise prediction intervals. The smoothing parameter ( $\lambda$ ) was calculated using generalized cross validation.

longer intervals between courtship and copulation. None of the other linear quadratic or correlational effects were significant.

Significance testing of eigenvalues from the canonical analysis using classical double regression indicated that the eigenvalue associated with the new multivariate axis  $m4$  was just significantly different from zero (Table 4). However, the more conservative permutation method, as advocated by Reynolds et al. (2010), indicated that this difference was likely to have been caused by random factors rather than nonlinear effects.

Of the 470 pairs in which the male was observed to court the female, 10 females did not copulate with the male during the observation period. These pairs comprised one full-sibling pair, one double first-cousin pair, two first-cousin pairs, two second-cousin pairs and four unrelated pairs.

#### Copulation Duration

None of the linear, correlational or quadratic coefficients for copulation duration were found to be statistically significant (Table 5). Significance testing of eigenvalues from the canonical analysis using classical double regression indicated one significant multivariate axis,  $m4$  (Table 6). However, again,  $P$  values calculated by the more conservative permutation method indicated that this difference was likely to have been caused by random factors.

**Table 3**

The four linear coefficients and matrix of four quadratic and six correlational nonlinear coefficients for the interval between courtship and copulation  $\pm$  SEs

	Linear ( $\beta$ )	Nonlinear ( $\gamma$ )			
		Relatedness	Female wing area	Sex comb	Male wing area
Relatedness	<b><math>-0.059 \pm 0.029</math></b>	$-0.020 \pm 0.038$	$0.012 \pm 0.030$	$-0.002 \pm 0.029$	$0.017 \pm 0.034$
Female wing area	$0.009 \pm 0.029$		$-0.024 \pm 0.022$	$-0.036 \pm 0.032$	$-0.007 \pm 0.025$
Sex comb	$-0.019 \pm 0.029$			$-0.018 \pm 0.025$	$-0.030 \pm 0.034$
Male wing area	<b><math>0.060 \pm 0.029</math></b>				$-0.029 \pm 0.019$

Significant results are shown in bold ( $P < 0.05$ ).

**Table 4**

Eigen-analysis for nonlinear effects of predictor variables on the interval between courtship and copulation

	$m1$	$m2$	$m3$	$m4$
Eigenvalue	0.005	$-0.039$	$-0.049$	$-0.100$
Double regression $P$	0.728	0.410	0.576	<b>0.047</b>
Permutation $P$	0.995	0.429	0.190	0.243
Canonical weights				
Relatedness	0.298	0.797	$-0.459$	$-0.254$
Female wing area	0.514	$-0.358$	$-0.578$	0.523
Sex comb	$-0.720$	0.275	$-0.300$	0.563
Male wing area	0.360	0.401	0.604	0.587

Significant result is shown in bold ( $P < 0.05$ ).

## DISCUSSION

Consistent with the results from the previous field study and the predictions of optimal inbreeding theory, our results indicate that *D. melanogaster* shows a preference for close relatives as mates. The negative linear effect of relatedness on the interval between courtship and copulation indicates that once courtship was initiated, flies took significantly less time to begin copulation when paired with more closely related individuals. There are two factors that may have contributed to this result. First, females may have preferred to mate with related males and accepted courtship from close relatives more quickly. Second, males may have shown a preference for related females and increased their courtship effort when presented with a more closely related female. We found no evidence for an effect of relatedness on either latency to first courtship or copulation duration. The lack of an effect of relatedness on these two largely male controlled traits would seem to suggest that the decrease in interval between copulation and courtship is more likely to be driven by female rather than male preference. This would be consistent with the earlier field study on *D. melanogaster* (Robinson et al. 2012) that detected a significant positive effect of relatedness between potential mates for male, but not female mating success. An asymmetry between male and female mate preference for relatedness is also consistent with the predictions of optimal inbreeding theory. In a system such as this, where females are the choosing sex and have a greater investment in offspring production, females may be expected to have optimal fitness at an intermediate level of inbreeding, whereas for males inbreeding should not be entirely avoided, but outcrossing always has the highest fitness (Purttinen 2011).

The results of this laboratory controlled study provide evidence that *D. melanogaster* shows a preference for close relatives as mates, and that this preference is likely to contribute to our finding that mating between close relatives was more common in the field than would be expected under random mating (Robinson et al. 2012). Together, these findings join a growing list of studies on a wide range of species that indicate a preference for close relatives as mates (e.g. Bateson 1982; Barnard & Fitzsimons 1988; Keane 1990;

**Table 5**The four linear coefficients and matrix of four quadratic and six correlational nonlinear coefficients for copulation duration  $\pm$  SEs

	Linear ( $\beta$ )	Nonlinear ( $\gamma$ )			
		Relatedness	Female wing area	Sex comb	Male wing area
Relatedness	$>0.000\pm 0.003$	$-0.006\pm 0.004$	$-0.004\pm 0.003$	$0.004\pm 0.003$	$0.001\pm 0.004$
Female wing area	$>0.000\pm 0.003$		$0.000\pm 0.002$	$0.000\pm 0.003$	$0.001\pm 0.003$
Sex comb	$0.004\pm 0.003$			$-0.001\pm 0.003$	$0.000\pm 0.004$
Male wing area	$-0.006\pm 0.003$				$0.002\pm 0.002$

Krokene & Lifjeld 2000; Cohen & Derhorn 2004; Kleven et al. 2005; Schjorring & Jager 2007; Thunken et al. 2007; Sherman et al. 2008; Richard et al. 2009; Wang & Lu 2011). As well as inclusive fitness benefits, some have suggested that preferences for related mates may be explained by a direct fitness advantage to the offspring of intermediate relatives resulting from a balance between factors such as inbreeding and outbreeding depression (Bateson 1983; Shields 1983; Waser & Price 1989). However, previous work on this population of *D. melanogaster* has found only costs to the fitness of offspring, over similar levels of relatedness to those investigated in the current study (Robinson et al. 2009, 2012), making direct fitness benefits an unlikely explanation for the observed preference in this case.

One prediction of optimal inbreeding theory that we did not find in our results was a preference for intermediate relatives. This would have been expected to be detected as a nonlinear effect of relatedness. We detected only a linear preference for increasing levels of relatedness over the range of levels of inbreeding assayed in our study. This indicates that either the optimal level of inbreeding was greater than or equal to crosses between full siblings, or that individuals show a preference for increased relatedness with no avoidance of extremely close relatives. This may not be surprising considering that in this population we previously found a standardized coefficient of inbreeding depression ( $b$ ) of between 0.311 and 0.453 for egg to adult viability (Robinson et al. 2009). Comparing this coefficient of inbreeding to the predictions of Puurtinen (2011), this suggests a stable level of inbreeding greater than that between full-sibling crosses.

Our results are in contrast to those of Ala-Honkola et al. (2011) who found no effect of relatedness on time to first copulation between full-sibling crosses in a laboratory population of virgin *D. melanogaster*. However, a likely explanation for our differing results is that the population used in their study had been maintained under laboratory conditions for over 200 generations prior to the assay. This is likely to have resulted in laboratory adaptation and may have resulted in reduced genetic diversity. Ala-Honkola et al. (2011) also found no evidence of inbreeding depression in egg to adult viability, in contrast to numerous studies looking at similar levels of inbreeding (e.g. Garcia et al. 1994; Mack et al. 2002; Robinson et al. 2009) and no evidence of postcopulatory inbreeding avoidance in contrast to a study by Mack et al. (2002) on a more

recently established laboratory population. Another contributing factor may have been that Ala-Honkola et al. (2011) did not distinguish between before and after the male started to court the female in their time to copulation measurement. Tan et al. (2012) also found no evidence of inbreeding avoidance in latency to copulation in a laboratory study of virgin *D. melanogaster*. However, they did find that males first mated to an unrelated female took significantly longer to mate with a second unrelated female than to a second related female. In our study, while females were virgin, males were most likely to have previously mated with a full-sibling female because they were allowed to remain in their original vial for several days after emergence. However, Tan et al. (2012) found no difference in latency to mate with a related or unrelated female after males were first mated with a related female.

In contrast to previous studies, which have found increased male size to be associated with increased mating success in both laboratory (Ewing 1961, 1964; Partridge & Farquhar 1983; Wilkinson 1987) and field situations (Partridge et al. 1987a; Markow 1988), our results suggest that female preference was for smaller males, with the interval between courtship and copulation decreasing with decreasing male size. However, most studies investigating mate choice in *D. melanogaster* have considered the outcome of competitive interactions, where male–male competition is likely to play a role. Indeed, there is evidence that larger males may be more successful at scramble competition and aggressive interactions (Partridge et al. 1987a, b). Furthermore, many studies suggest the increased mating success of large males is more likely to be the result of male interactions than female preference (Partridge et al. 1987b; Wilkinson 1987), although examples of increased mating success of large males have also been found using no-choice experimental designs (Partridge & Farquhar 1983). A possible explanation for why females might show a preference for smaller males is the harm caused to females by male manipulation through accessory gland proteins (Ram & Wolfner 2007). The harm caused to females has been found to increase with increasing male size, such that females mating with larger males suffer reduced lifetime reproductive success (Pitnick & Garcia-Gonzalez 2002; Friberg & Arnqvist 2003). There is also evidence that the indirect benefits of mating with more costly males may not make up for the extra costs to females (Hosken & Tregenza 2005; Stewart et al. 2005).

Our results also differ to those of Lefranc & Bundgaard (2000) who found that both male and female body size influenced copulation duration. One explanation for this difference is that Lefranc & Bundgaard (2000) created variation in body size experimentally by varying the degree of crowding (starvation) among larvae. In our experiment, larval densities were kept low and relatively constant, which would have resulted in far less variation in body size. Consistent with our previous field study of this population of *D. melanogaster*, we did not find evidence of directional selection on sex comb tooth number (Robinson et al. 2012). However, in both studies, multivariate analysis suggested that sex comb tooth number might be involved in a more complex relationship with mating success.

**Table 6**

Eigen-analysis for nonlinear effects of predictor variables on copulation duration

	m1	m2	m3	m4
Eigenvalue	0.025	0.009	-0.041	-0.046
Double regression $P$	0.415	0.578	0.970	<b>0.037</b>
Permutation $P$	0.886	0.736	0.175	0.513
Canonical weights				
Relatedness	0.134	0.343	-0.095	0.925
Female wing area	-0.532	-0.640	-0.487	0.265
Sex comb	0.112	0.442	-0.849	-0.268
Male wing area	-0.829	0.526	0.183	-0.056

Significant result is shown in bold ( $P < 0.05$ ).

The no-choice trials used in this experiment are useful in looking at mate choice without the complicating factors of male–male interactions. A no-choice scenario is, however, unlikely to represent the usual field conditions for *D. melanogaster*. For example, Markow & Sawka (1992) found that females were courted by an average of five males simultaneously. Mate encounter rates and whether choice is made sequentially or simultaneously are expected to influence potential inclusive fitness benefits from mating with a close relative (Kokko & Ots 2006). Future work may focus on more complex, competitive scenarios, representative of field conditions and may help to determine whether the apparent mate preference for relatedness observed in this study is likely to explain the increased chance of mating between close relatives observed in the field population.

The theory of optimal inbreeding for inclusive fitness benefits represents a major shift in the way we think about inbreeding and may have wide-reaching evolutionary consequences (Puurtinen 2011). To give an example, if individuals in a small or fragmented population show a preference for mating with relatives, there may be more inbreeding and resulting loss of fitness from inbreeding depression than would otherwise be anticipated. This may be of great concern in the context of conservation of natural populations. On the other hand, increased levels of inbreeding in populations may lead to more efficient purging of genetic load, as more deleterious recessives are exposed to selection. This could result in species that practise optimal inbreeding suffering lower costs of inbreeding where it occurs. It is clear that more research is needed on optimal inbreeding, both in determining how widespread preferences for relatives are, and in investigating the effects such preferences are likely to have on demographic and evolutionary processes.

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