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Effects of inbreeding on aversive learning in *Drosophila*

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Abstract

Inbreeding adversely affects life history traits as well as various other fitness-related traits, but its effect on cognitive traits remains largely unexplored, despite their importance to fitness of many animals under natural conditions. We studied the effects of inbreeding on aversive learning (avoidance of an odour previously associated with mechanical shock) in multiple inbred lines of *Drosophila melanogaster* derived from a natural population through up to 12 generations of sib mating. Whereas the strongly inbred lines after 12 generations of inbreeding ($0.75 < F < 0.93$) consistently showed reduced egg-to-adult viability (on average by 28%), the reduction in learning performance varied among assays (average = 18% reduction), being most pronounced for intermediate conditioning intensity. Furthermore, moderately inbred lines ($F = 0.38$) showed no detectable decline in learning performance, but still had reduced egg-to-adult viability, which indicates that overall inbreeding effects on learning are mild. Learning performance varied among strongly inbred lines, indicating the presence of segregating variance for learning in the base population. However, the learning performance of some inbred lines matched that of outbred flies, supporting the dominance rather than the overdominance model of inbreeding depression for this trait. Across the inbred lines, learning performance was positively correlated with the egg-to-adult viability. This positive genetic correlation contradicts a trade-off observed in previous selection experiments and suggests that much of the genetic variation for learning is owing to pleiotropic effects of genes affecting functions related to survival. These results suggest that genetic variation that affects learning specifically (rather than pleiotropically through general physiological condition) is either low or mostly due to alleles with additive (semi-dominant) effects.

Introduction

Inbreeding arises through mating between relatives and results in increased homozygosity (Wright, 1921; Crow & Kimura, 1970; Rumball *et al.*, 1994). Inbreeding typically leads to a decline in fitness-related traits, such as survival, competitive ability, viability, fertility, pathogen resistance. (Wright, 1977; Latter & Sved, 1994; Latter *et al.*, 1995; Crnokrak & Roff, 1999; Bader *et al.*, 2000; Keller & Waller, 2002; Acevedo-Whitehouse *et al.*, 2003; Lyons *et al.*, 2009), a phenomenon known as inbreeding depression (Charlesworth & Charlesworth, 1987; Falconer, 1989). Avoidance of mating with kin, observed in many species (Pusey & Wolf, 1996; Weisfeld *et al.*, 2003), suggests that inbreeding depression under natural conditions is strong enough to cause selection for mechanisms that prevent inbreeding.

Two major hypotheses could explain inbreeding depression (Charlesworth & Charlesworth, 1987): overdominance (Schull & Neel, 1972), which gives a fitness advantage to heterozygous individuals, and (directional) dominance (Davenport, 1908; Wright, 1977), whereby the increase in homozygosity reveals the effects of

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recessive deleterious alleles. These two mechanisms are not mutually exclusive, different patterns exist in different species (Ziehe & Roberds, 1989; Brewer *et al.*, 1990; Roff, 2002). However, the dominance hypothesis is better supported empirically, at least in *Drosophila*, mice and humans (Deng *et al.*, 1998).

In contrast to life history, the effects of inbreeding on behavioural and in particular cognitive traits remain poorly known. Among the few existing studies, inbreeding is suggested to cause deficits in parental behaviour (Margulis & Altmann, 1997; Margulis, 1998) and copulatory behaviour (Dewsbury *et al.*, 1979) in mice. It is also suggested to affect male courtship behaviour in the housefly (Meffert & Bryant, 1991), decrease male mating behaviour in fish (Mariette *et al.*, 2006; Ala-Honkola *et al.*, 2009) and butterflies (Joron & Brakefield, 2003) and reduce song repertoire in male song sparrow (Reid *et al.*, 2005). The effects of inbreeding on learning ability have been examined in rats, where inbred strains showed a significantly lower result in spatial learning (Harker & Whishaw, 2002); however, the control outbred strains for that study were derived from a different genetic background. In human populations, correlative studies have found inbreeding to be deleterious to some cognitive functions, like reading or learning ability (Bashi, 1977; Afzal, 1988; Rudan *et al.*, 2002; Abu-Rabia & Maroun, 2005), but these finding are not universal (Neel *et al.*, 1970). Moreover, interpretation of these correlative studies can be confounded by other factors, including socio-cultural differences. Marriage between relatives is likely to depend on socio-economic background, which may also affect the results of cognitive performance tests.

Here, we study the effects of experimental inbreeding on a cognitive trait – associative learning ability – in *Drosophila melanogaster*. *Drosophila* are capable of learning in response to classical associative conditioning, as well as in operant conditioning (involving motor responses and decision-making; Quinn *et al.*, 1974; Kawecki & Mery, 2006;). Four memory types have been identified: short term (STM), middle term (MTM), long term (LTM) and a form of consolidated memory named anaesthesia-resistant memory (Quinn & Dudai, 1976; Tully *et al.*, 1994; Isabel *et al.*, 2004), which does not involve protein synthesis (Waddell & Quinn, 2001). It has been shown that flies are also able to store information about various features, like visual cues (Peng *et al.*, 2007), food (Tempel *et al.*, 1983), egg-lying sites (Mery & Kawecki, 2002) and conspecifics, like mates (Siegel & Hall, 1979) or competitors (Yurkovic *et al.*, 2006). As in most species, inbreeding in flies results in deterioration in fitness-related traits, such as competitive ability, viability, fecundity and male mating success (Castle, 1906; Mackay, 1985; Miller *et al.*, 1993; Latter & Sved, 1994; Hughes, 1995; Latter *et al.*, 1995). Among behavioural traits, inbreeding depression affects male song frequency in *Drosophila montana* (Aspi, 2000) and reduces male mating ability in *D. melanogaster* (Sharp, 1984; Miller *et al.*, 1993). Moreover, artificial selection for improved learning ability performed on small populations actually led to a decline in learning performance, presumably because of inbreeding depression (Hewitt *et al.*, 1983).

Positive responses to experimental selection on learning performance in other experiments (Lofdahl *et al.* 1992; Mery & Kawecki, 2002; Reif *et al.*, 2002; Mery *et al.*, 2007b; Dunlap & Stephens, 2009) show that *Drosophila* populations harbour natural genetic variation in learning ability; a specific natural polymorphism contributing to this variation has been identified (Mery *et al.*, 2007a; Kaun *et al.*, 2008). Correlated responses to selection revealed negative additive genetic correlations of learning performance with larval competitive ability and adult lifespan, presumably reflecting evolutionary trade-offs (Mery & Kawecki, 2003; Burger *et al.*, 2008; Kolss & Kawecki, 2008). To gain insights into the genetic architecture of learning ability, we used multiple inbred lines of *Drosophila* derived by sib mating from a base population recently acquired from the field. We ask the following questions.

First, does learning performance show inbreeding depression, and how strong is it, compared to inbreeding depression for egg-to-adult viability, for which inbreeding depression is firmly established (Mackay, 1985)? Inbreeding depression would indicate that polymorphisms affecting learning performance segregate in the base population, and that the alleles that reduce learning are, on average, recessive, partially recessive or overdominant.

Second, is there variation among the inbred lines, and do all of them show inferior learning performance and viability compared to the outbred base population? Because different inbred lines become randomly fixed for different alleles, variation among inbred lines captures a part of the genetic variation present in the base population. Variation among inbred lines would help to interpret potential absence of inbreeding depression as reflecting additivity of allelic effects (i.e. semi-dominance) rather than lack of genetic variation in the base population. Furthermore, if there were on average some inbreeding depression, but some of the inbred lines were equal or superior to the outbred population, it would indicate that heterozygosity is not required for high learning ability. This would support the dominance rather than the overdominance hypothesis main mechanism of inbreeding depression.

Third, does learning performance of individual inbred lines correlate with their egg-to-adult viability? Such correlation would suggest pleiotropy. A positive correlation would suggest that inbreeding depression is mostly caused by alleles that impair some general functions of the organism affecting both life history and learning performance. On the contrary, a negative correlation between the fitness components and learning performance would suggest a trade-off, similar to trade-offs between learning and competitiveness (Mery & Kawecki,

2005) or lifespan (Burger *et al.*, 2008) revealed by selection experiments.

Fourth, is there evidence for purging of alleles that reduce learning? Purging of recessive alleles that impair learning might occur if they also impair fitness under the experimental conditions, leading to selective loss of some lines. Under purging, estimates of inbreeding depression from early generations (before line loss) are expected to be larger than estimates from surviving lines later in the experiment. Purging should also result in F_1 crosses between inbred lines showing on average superior learning performance compared to the base population (Crnokrak & Barrett, 2002).

Material and methods

We first describe how the inbred lines and the outbred controls were derived. In the subsequent sections, how the phenotypes were assayed, and how they were used to assess inbreeding depression, performance of crosses and variation among inbred lines.

Base population and inbred lines

The base population originated from 400 flies collected in Valais (Switzerland), in October 2007. It was maintained in a large population cage at the size of about 1200 adults and a generation time of 3 weeks on a yeast-cornmeal medium (David & Clavel, 1965), at 25°C, 60% humidity and 12 : 12 h light : dark cycle. The inbred flies were raised the same way except for the density of population.

Inbred lines were produced by sib mating. A mated female was isolated and allowed to oviposit. Her offspring were then allowed to mate among themselves upon emergence, and a new mated female was isolated and used to establish the next generation. Multiple mating is common in *Drosophila* (Milkmann & Zeitler, 1974; Imhof *et al.*, 1998), and thus, the offspring of a randomly chosen mated female may have several fathers, allowing for the possibility of half-sib rather than full-sib mating in our experiment. The coefficients of inbreeding F were thus bound by the following recurrence equations (Ollivier, 2002):

$F_{t+1} = 1/4 \quad (1 + F_{t-1} + 2F_t)$ (assuming full-sib mating, maximum inbreeding)

$F_{t+1} = 1/8 \quad (1 + 6F_t + F_{t-1})$ (assuming half-sib mating, minimum inbreeding)

To compensate for the anticipated loss of lines owing to fixation of highly deleterious alleles, we initially established 50 parallel lines. After 12 generations of sib mating, the surviving 15 inbred lines were expanded to around 50–100 individuals and subsequently maintained at this size to reduce losses because of demographic stochasticity. By that time, the expected inbreeding coefficient was between 0.75 (assuming all matings were between half sib) and 0.93 (assuming all mating begin full sib); with 50% of each type of mating F would be 0.88.

Many of the original inbred lines were lost in the course of inbreeding, and this process was unlikely to be random with respect to viability effects of alleles being fixed, leading to some purging of such deleterious alleles (Crnokrak & Barrett, 2002). Through pleiotropic effects of genes affecting line loss, such purging might have also affected the observed inbreeding depression for learning performance. Therefore, at a later stage, we independently derived additional 15 inbred lines from the same base population. These 'moderately inbred lines' were obtained by two generations of full-sib mating ($F = 0.38$) under the same environmental conditions as described earlier. Full-sib mating was ensured by isolating virgin females and subsequent controlled mating with a single randomly selected male. None of these additional lines were lost, so they are more representative of the base gene pool. Their viability and learning performance were compared to the original highly inbred lines in a simultaneous (cross-sectional) assay.

Phenotypic assays

Learning performance

Flies for the learning assays were raised from eggs laid in mass oviposition during 2 days in 200-mL vial containing 30 mL of standard food. When needed (inbreeding depression and crosses experiments, see below), the emerging adults were anesthetized with CO_2 and mixed, then separated in groups of 60 flies, in 60-mL vials containing 10 mL of food. If CO_2 was used, the flies had at least 24 h to recover before being assayed.

The learning assay involved an association between an odour (conditioned stimulus) and an aversive mechanical shock (unconditioned stimulus; Kawecki & Mery, 2006). Flies were conditioned and tested in groups of around 60 individuals (sexes mixed), aged 7–10 days. Conditioning consisted of one or several conditioning cycles. In each conditioning cycle, the group of flies was first exposed for 30 s to one odour (the conditioned stimulus) and simultaneously subject to mechanical shock delivered by a test tube shaker (1 s of shocks every 5 s), followed by 60 s humid air flow, 30 s of the second odour (the neutral stimulus); another 60 s period of humid air flow completed the conditioning cycle. When several conditioning cycles were used (to increase the total exposure to conditioning), they immediately followed one another. Octanol and 4-methyl-cyclohexanol (MCH) dissolved in paraffin (0.6 mL per litter of paraffin) were alternately used as conditioning and neutral stimulus. Both odours are innately avoided by the flies.

A set time after the end of conditioning, the flies were placed in a T-maze and allowed to choose between the odours for 45 s. To obtain an estimate of preference, the flies in each arm of the T-maze were counted; flies

remaining in the central chamber of the T-maze were ignored. The assays were paired; each group of flies conditioned to avoid octanol was paired with a group conditioned to avoid MCH. One learning score was calculated for each such pair, as the difference in the proportion of flies choosing octanol between the group conditioned to avoid MCH and the group conditioned to avoid octanol. Learning scores were then used as dependent variable in ANOVA after checking for homogeneity of variance (Bartlett test) and normality of residues (visually controlled with Q–Q plot).

Unconditioned responses to odours

The response to odours (odour avoidance) without prior conditioning (i.e. in naïve flies) was also measured. The flies were subjected to the same pattern of shock as in the conditioning procedure, but without exposure to odours. They were then transferred to the T-maze and allowed to choose between one odour (octanol or MCH) and the solvent (paraffin oil). The proportion of flies choosing the solvent indicates their innate tendency to avoid the odour.

Egg-to-adult viability

Eggs were collected in mass oviposition on fruit jelly overnight. One hundred eggs were transferred to a 60-mL vial containing 10 mL of food; eggs that were infertile (transparent) or mechanically damaged were eliminated. In some cases, some lines did not lay enough eggs, in which case the vials were set up with fewer than 100 eggs (see below). To assess viability, we counted the number of adults that emerged within 9 days (normal food) or within12 days (poor food), counting from emergence of the first fly. The proportion of eggs that resulted in an emerged adult was used as an estimate of viability (one value per vial).

Inbreeding depression

General design

The inbred lines were assessed for inbreeding depression after five generations of inbreeding (viability), after eight generations (viability and preliminary assessment of learning performance) and after twelve generations of inbreeding (viability, detailed assessment of learning performance and unconditioned odour responses). Viability tests and a restricted set of learning performance tests were also carried out for the independently derived 'moderately inbred lines' (see above). Theses lines were assessed in a 'cross-sectional' experiment in parallel with the 'strongly inbred lines' (12 generations of inbreeding) and with the outbred controls.

Learning performance

Inbreeding depression is quantified as the proportional reduction in mean performance of inbred individuals. Learning assays were performed in groups of 60 adults (see above). Rather than forming each group using a single inbred line, we mixed equal numbers of adults from each inbred line, and the groups of 60 flies were derived from this mixed population. This was done to reduce the variance among the replicates and thus to increase the precision of the mean estimate while not exceeding the number of replicates that could technically be handled. This allowed us to study the average effect of inbreeding on learning performance under a varying number of conditioning cycles (memory acquisition) and a range of time between conditioning and test (memory decay). In all assays described below, the outbred flies from the base population served as controls.

The first assay was performed after eight generations of inbreeding; flies originating from 24 inbred lines were assayed for 20 min memory after 2 conditioning cycles. After the inbred lines completed 12 generations of brother–sister mating, we performed more extensive assays. They included the following:

(A) The acquisition of short-term memory: the learning scores were assayed about 4 (range 2–6) min after a varying number (1–5) of conditioning cycles.
(B) The acquisition of middle-term memory: the learning scores assayed 60 min after 1–3 conditioning cycles.
(C) The memory decay: the learning scores assayed after 5 conditioning cycles as a function of the interval between conditioning and test (5 min, 1, 4, 19 h).

Assay (B) was performed immediately after the 12 generations of inbreeding were completed and included flies from 20 inbred lines surviving at this point. Five of these lines were subsequently lost, and assays (A) and (C) were carried out on flies originating from the remaining 15 inbred lines.

Finally, we compared the learning performance of flies from 15 highly inbred flies ($0.75 < F < 0.93$), 15 moderately inbred lines ($F = 0.38$) and outbred flies in a single 'cross-sectional' experiment. We assessed their short-term memory after 3 conditioning cycles, which was the measure of learning performance that showed most pronounced inbreeding depression in the other experiments.

The learning scores were subject to an ANOVA, with inbreeding status and, where applicable, number of conditioning cycles or time between conditioning and testing treated as categorical fixed factors. Where applicable, the initial model also included the interaction between the fixed factors; if not significant, this interaction was removed from the final model reported in the Results. Some of the experiments were performed over two or more experimental sessions, treated as random blocks. We only mention block effects when they were significant; the same applies to interactions between block and other factors. Nonsignificant block interactions were taken out from the model.

Unconditioned responses to odours

To see whether the effects of inbreeding on learning could have been confounded by differences in unconditioned odour responses, we also studied the effect of inbreeding on the responses to odours (odour avoidance) of naïve flies as described earlier. This was performed after 12 generations of inbreeding on flies originating from 14 inbred lines, mixed as for the learning assay, as well as on outbred flies. The proportion of flies choosing the solvent was treated as a dependent variable in an ANOVA, with inbreeding status and odorant as fixed factors and block (experimental session) as a random factor.

Egg-to-adult viability

To estimate the inbreeding depression in egg-to-adult viability, three different experiments were conducted. Experiment 1 compared inbred flies from 40 lines after five generations of sib mating ($0.5 < F < 0.67$, 40 lines), to the outbred base population ($N = 10$ vials). In experiment 2, flies from 20 lines remaining after eight generations of sib mating ($0.61 < F < 0.83$) were compared with the outbred base population ($N = 5$ vials). In these two experiments, each vial in the inbred treatment was set up with a mix of eggs from four lines, each contributing 25 eggs to the total of 100. Different sets of four lines were used to set up each vial, and all lines were equally represented in the experiments. The data of these two experiments were analysed with a Mann–Whitney test comparing inbred and outbred flies. In experiment 3, the viability of highly inbred lines (12 generations of sib mating, $0.75 < F < 0.93$) was compared to that of outbred flies as well as to moderately inbred flies ($F = 0.38$). In this experiment, each vial was set up with 100 eggs from a single line, with 2–4 vials for each of 12 highly inbred and 14 moderately inbred lines and 30 vials with the outbred flies. The data from experiment 3 were analysed with a generalized linear model with quasi-binomial error to correct for overdispersion.

Crosses between inbred lines

To assess whether purging of alleles that impair learning performance or viability had occurred during the inbreeding process, we assessed the average learning performance and egg-to-adult viability of three types of flies: our highly inbred lines, F_1 crosses between flies from different highly inbred lines and the outbred flies from the base population. The parents of all the animals used in these experiments were raised under standard conditions. To obtain the crosses, 14 highly inbred lines (12 generations of sib mating) were crossed in a circular scheme, with line 1 crossed with line 2, line 2 with line 3, …, line 14 with line 1; each line thus provided the dams for one cross and sires for another. For each cross, eggs were collected from five females and five males; this corresponded to the number of virgin females available from the least productive inbred line. The inbred and outbred flies were raised the same way. The individuals tested for egg-to-adult viability and learning were produced from the same parents.

For learning performance, equal numbers of flies from the 14 inbred lines were combined to create a mixed inbred population; adults from the 14 crosses were likewise combined to obtain a mixed F_1 population. These two populations and the outbred population were then assayed for short-term memory after 3 cycles of conditioning, as well as for unconditioned responses to odours. The learning scores were analysed with an ANOVA, with inbreeding status (outbred, inbred and crossed) as the fixed factor and block (defined by three experimental sessions) as a random factor. The odour avoidance scores were likewise analysed with an ANOVA, treating inbreeding status and odorant as fixed effects and experimental session as a random block effect.

We also measured the egg-to-adult viability of the three categories of flies (inbred, crosses and outbred) on normal food, as well as on poor food containing 10% of yeast used in normal food. Within each category, the eggs were randomly distributed among vials, each vial set up with eggs from up to four lines, according to egg availability. Three of the 14 inbred lines did not produce enough eggs for this assay, and some other lines had poor fertility, so the target 100 eggs per vial not always could be reached. Specifically, on normal food, 32% of the vials contained between 75 and 100 eggs, and 11% fewer than 75. On poor food, 17% of the vials contained between 75 and 100 eggs, and 8% fewer than 75. For each vial, viability was calculated as the number of adult flies emerged per vial divided by the number of eggs originally placed in this vial. These values were subject to an ANOVA, with inbreeding status and food type and their interaction as fixed effects.

Variation among inbred lines

Learning performance

Because of the labour intensity of the learning assays, for the variation among inbred lines we concentrated on the learning assay for which the average effect of inbreeding was most pronounced, that is, on short-term memory after 3 cycles of conditioning. This was performed on 14 highly inbred lines (12 generations of inbreeding $0.75 < F < 0.93$), with 9–14 replicate learning scores per line.

Egg-to-adult viability

Fourteen inbred lines were included, with 4–7 replicate vials per line with 100 eggs each. Some lines had poor fertility, so ten vials (out of 97) contained fewer than the target 100 eggs (15–90 eggs; one vial in line 35, two in line 13, five in line 14 and two in line 48). Seven replicates from the outbred base population were also included. The viability of the flies was assessed as described earlier.

The learning scores and viability values were checked for homogeneity of variance (with Bartlett's test) and normality of residuals (visually controlled with normal probability Q–Q plot). One-way ANOVA with inbred line as the (random) factor was used to estimate the among-line variance component and test for its significance. Additionally, each line was compared to the outbred population with Dunnett's test. For each line, we also used a *t*-test with the null hypothesis that its mean learning score is zero. Finally, the normality of the distribution of line means was tested with Anderson–Darling normality test.

Results

Inbreeding depression

Learning performance

After eight generations of brother–sister mating, the inbred flies tended to show only slightly poorer short-term memory (learning score 20 min after 2 conditioning cycles 0.59 ± 0.04) than the outbred controls (0.64 ± 0.03; mean ± SE; $F_{1,20} = 1.2$, $P = 0.28$, $N = 11$).

More extensive assays carried after 12 generations of brother–sister mating provided more convincing evidence of inbreeding depression affecting learning. Specifically, for short-term memory acquisition (Fig. 1a), inbred flies showed significantly lower learning scores than outbred flies (ANOVA, $F_{1,82} = 13$, $P = 0.0005$). The effect was more pronounced for intermediate conditioning intensity (2–4 conditioning cycles), although the interaction between inbreeding status and cycle number was not significant ($F_{4,78} = 0.64$, $P = 0.63$; the interaction was eventually removed from the model). A similar result was observed for middle-term memory (Fig. 1b), where the inbred flies also performed less well than outbred ($F_{1,75} = 5.46$, $P = 0.02$), with the effect most pronounced after 2 conditioning cycles, even though the interaction between inbreeding status and cycle number was again not significant ($F_{2,73} = 0.74$, $P = 0.48$). In a separate memory decay experiment (Fig. 1c), we detected no effect of inbreeding on memory after 5 conditioning cycles and the way it declined with time between conditioning and testing (inbreeding status: $F_{1,55} = 0.23$, $P = 0.63$; inbreeding × time interaction: $F_{3,52} = 0.18$, $P = 0.91$, removed from the final model). There was no block effect ($F_{1,55} = 0.48$, $P = 0.5$), but the block × time interaction was significant ($F_{3,55} = 3.59$, $P = 0.02$). As expected, the learning scores declined after 1 h (time between conditioning and test: $F_{3,55} = 94.40$, $P < 0.0001$), although more abruptly than expected, so that the learning scores after 4 and 19 h were not distinguishable from zero. The short-term memory learning scores after 5 conditioning cycles in the experiments presented in Fig. 1a, c did not differ significantly between experiments ($F_{1,33} = 2.43$, $P = 0.13$).

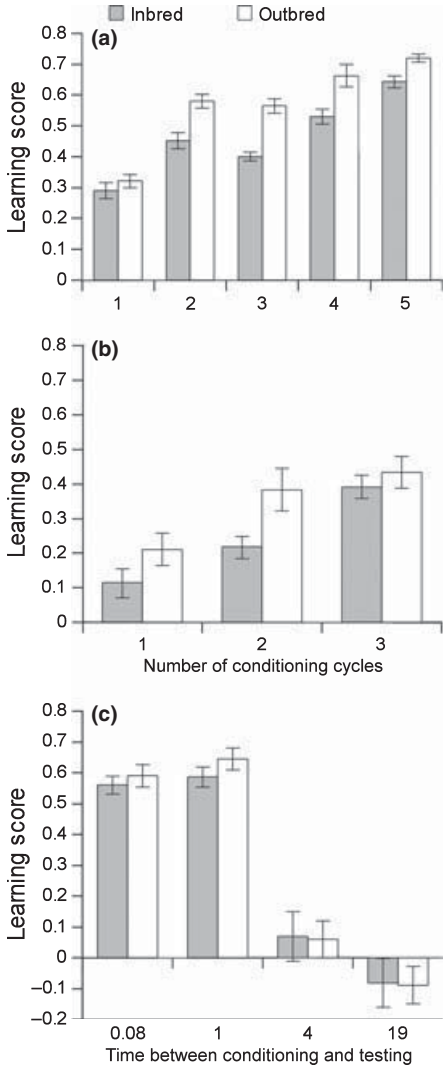

Fig. 1 Effects of 12 generations of sib mating on learning performance. (a) Acquisition of short-term memory as a function of the number of conditioning cycles (N = 8–10 learning scores per bar). (b) Acquisition of middle-term memory (N = 16 per bar for 1 and 3 cycles, and 8 for 2 cycles). (c) Memory decay: learning score after 5 conditioning cycles as a function of time between conditioning and test (N = 8 per bar).

Finally, we did a cross-sectional study including, in addition to the outbred and the highly inbred flies, also moderately inbred flies subject to two generations of full-sib mating ($F = 0.38$). We assayed these flies for short-term memory after 3 conditioning cycles, under the conditions that previously allowed us to detect inbreeding depression for learning (Fig. 1a). Yet in this experiment both highly (mean learning score ± SE: 0.55 ± 0.03) and moderately inbred (0.52 ± 0.03) flies only tended to be slightly inferior to the outbred flies (0.61 ± 0,03; $F_{2,45} = 2.18$, $P = 0.12$, $N = 16$). Averaged over all assays on lines subject to 12 generations of sib

mating, the inbreeding depression for learning performance (the proportional reduction in the learning score) was about 18%.

Unconditioned responses to odours

Inbreeding did not affect the response to odours (ANOVA, $F_{1,28} = 0.11$, $P = 0.74$; block effect, $F_{1,28} = 18.83$, $P = 0.0002$). Both odours were avoided, octanol slightly more (ANOVA, $F_{1,28} = 20.26$, $P = 0.0001$). These results indicate that inbred and outbred flies had the same olfactory response in the absence of conditioning, and thus the inbreeding effects on learning performance reported earlier were not because of decreased odour detection abilities of the inbred flies.

Egg-to-adult viability

In contrast to learning, the evidence for inbreeding depression for viability was unambiguous in all three experiments (Fig. 2; experiment 1: $W = 0$, $P = 0.01$; experiment 2: $W = 0$, $P = 0.0002$; experiment 3, GLM: $\chi^2 = 89.9$, d.f. = 2, $P < 0.0001$). Averaged over the three experiments, 12 generations of sib mating led to 28% reduction in viability.

Crosses between inbred lines

Analysis of crosses between inbred lines revealed no evidence that deleterious alleles had been purged during the course of inbreeding. In contrast to the prediction of the purging hypothesis, the viability of the crosses was intermediate between inbred and outbred flies (Fig. 3a; ANOVA $F_{2,86} = 31.5$, $P < 0.0001$, Tukey test $P < 0.05$). Even though, as expected, viability was lower on poor food ($F_{1,86} = 25.1$, $P < 0.0001$), differences among the three treatments were similar (interaction $F_{2,84} = 0.079$, $P = 0.92$, removed from the model).

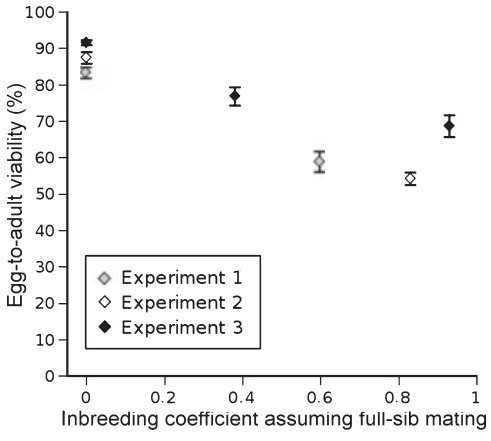

Fig. 2 Effects of inbreeding on the egg-to-adult viability (percentage of fertile eggs that survived to adulthood), plotted as a function of maximum inbreeding coefficient. The results stem from three separate experiments, for details see Material and methods.

The learning performance of the crosses was tested in the assay, for which the results reported earlier indicated most pronounced inbreeding depression: short-term memory after 3 conditioning cycles (compare Fig. 1b). Their learning performance in this assay was indistinguishable from that of the outbred population (Fig. 3b). The confidence interval of difference between crosses and outbred is narrow (−0.06,0.06). This experiment also confirmed that inbreeding depression for learning performance was weak: the inbred lines had only slightly lower learning scores than the outbred lines and crosses; the difference was only significant if the outbred and crossed treatments were pooled ($F_{1,61} = 4.8$, $P = 0.032$). There were also significant differences among the three blocks, in which the experiment was carried out (ANOVA, $F_{2;61} = 13.2$, $P < 0.0001$).

For odour avoidance, crosses between inbred lines did not differ from the outbred base population ($F_{1,27} = 0.06$, $P = 0.8$; Fig. 4). Both odours were avoided, octanol significantly more than MCH, just as in the other experiments ($F_{1,27} = 17.83$, $P = 0.0002$; interaction

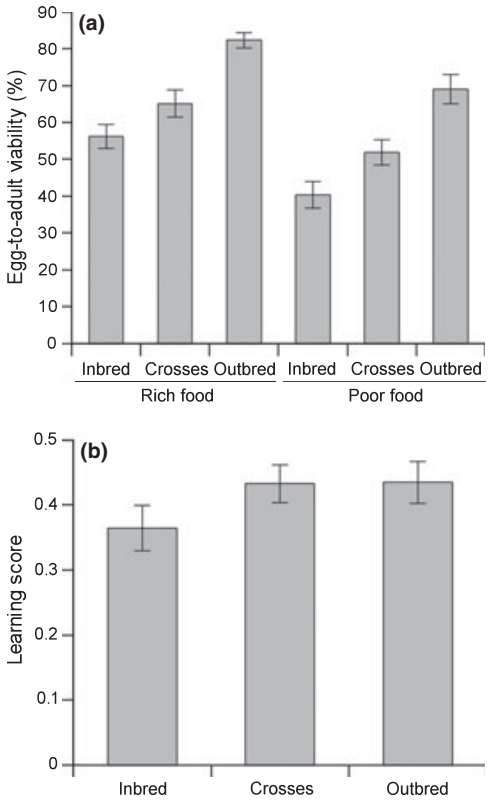

Fig. 3 Comparison of strongly inbred lines (12 generations of sib mating; $0.75 < F < 0.93$), crosses between inbred lines and outbred base population. (a) Egg-to-adult variability on normal and poor food, $N = 12$ vials per food level for inbred, 16–17 per food level for crosses and outbred. (b) Learning ability; $N = 21$–22 learning scores per bar.

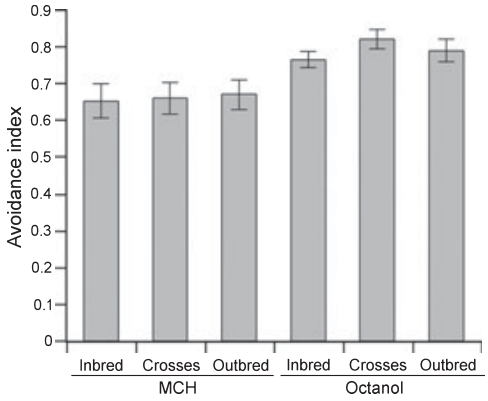

Fig. 4 Unconditioned response to odours: the proportion of flies choosing solvent over the odorant (octanol or methyl-cyclohexanol).

inbreeding status × odour $P = 0.6$; the interaction was removed from the final model).

Variation among inbred lines

After 12 generations of inbreeding, we also tested each line separately to study the variation of learning performance among the lines and its relationship with egg-to-adult viability. Learning performance turned out to be positively correlated across lines with their egg-to-adult viability (Fig. 5a; Pearson's $r = 0.63$, d.f. = 12, $P = 0.015$). The inbred lines varied substantially with respect to both learning performance ($F_{13,149} = 3.67$, $P < 0.0001$) and egg-to-adult viability ($F_{13,76} = 14.8$, $P < 0.0001$). The normal probability plot (Fig. 5b) indicates that the line means of the learning scores fitted the normal distribution almost perfectly (Anderson–Darling normality test, $A = 0.1083$, $P = 0.99$). The corresponding means for viability also did not deviate from normal distribution ($A = 0.313$, $P = 0.51$). Except for one (line 17, $t = 2.0247$, d.f. = 8, $P = 0.077$), all the inbred lines had learning scores significantly greater than zero. According to Dunnett's test, only two lines had significantly worse learning scores than the outbred ($P < 0.05$). In contrast, the majority of lines were inferior to the outbred for egg-to-adult viability ($P > 0.05$). Variance among the lines accounted for 77% of variance in learning scores and 94% of variance in egg-to-adult viability values. It should, however, be noted that each replicate was based on 100 individuals, so the within-line among-replicate component underestimates the variation among individual flies within lines. The genetic coefficient of variation (square root of among-line variance divided by mean of the trait, Houle, 1992) was 0.68 and 0.82 for learning and viability, respectively. Inbreeding depression could also be calculated for each line separately; the coefficient of variation of this line-specific inbreeding depression (square root of variance among lines divided by the

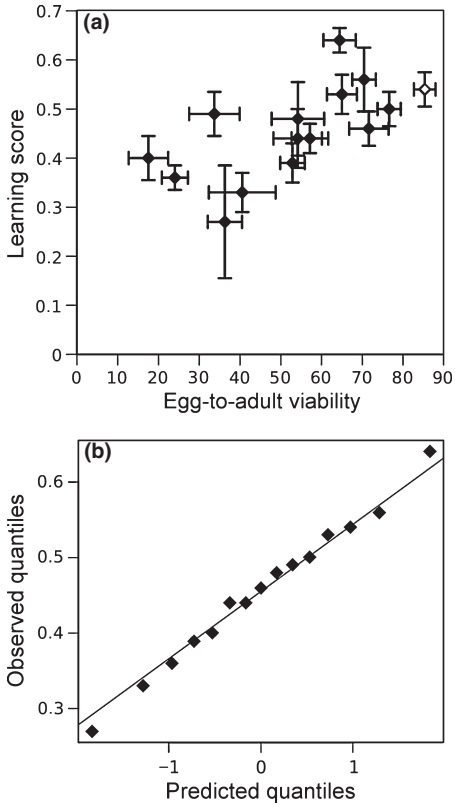

Fig. 5 Variation among inbred lines. (a) Mean egg-to-adult viability and short-term memory values of individual inbred lines (filled symbols), compared to the outbred base population (open symbol). Bars indicate one standard error. (b) The normal probability plot of inbred line means of short-term memory learning score; the close correspondence between the predicted and observed quantiles indicates a good fit of line means to the normal distribution.

mean inbreeding depression) was 1.01 and 0.54 for learning and viability, respectively.

As an alternative estimator of inbreeding depression, we also calculated the mean and standard error of inbred lines (mean ± standard error) and outbred base population (learning: inbred lines 0.44 ± 0.02, outbred population 0.54 ± 0.04; viability: inbred lines 51.32 ± 4.9, outbred population 85.29 ± 2.65).

Discussion

Our study confirms that inbreeding adversely affects egg-to-adult viability in *Drosophila* (Dobzhansky & Spassky, 1962; Biemont, 1976, 1978). Twelve generations of sib mating reduced viability by about 30%; the effect tended to be even more pronounced on poor food (40%). Only 15 of the initial 50 inbred lines survived to the 12th generation, and it is probable that the survival of lines was positively correlated with larval viability. It is thus likely that the observed inbreeding depression considerably underestimates the overall effect of inbreeding on

viability, even though we did not detect any direct evidence for purging of deleterious alleles (see below). A substantial viability reduction was already observed after two generations of full-sib mating. This confirms results from other studies which demonstrated strong inbreeding depression for fitness-related traits in *Drosophila* (reviewed in Charlesworth & Charlesworth, 1999).

The quantitative effect of inbreeding on learning performance varied among our experiments. This, together with differences in learning performance observed between blocks within experiments, is consistent with the general observation that behavioural and in particular cognitive traits are highly labile and sensitive to uncontrollable environmental variation. Nonetheless, all experiments showed at least a tendency for learning performance to be reduced in inbred compared to outbred flies. Because we observed a positive correlation between learning performance and viability across inbred lines, and because many lines were lost in the course of inbreeding, it is possible that with the least viable lines also the lines with the lowest learning performance went extinct. This would have led to an underestimation of the effect of inbreeding on learning, although perhaps to a lower degree than for viability because inadvertent selection during inbreeding may have acted directly on viability but only indirectly (via the positive correlation with viability) on learning. However, the inbreeding depression for learning observed in the additional set of moderately inbred lines ($F = 0.38$) is of similar magnitude as for the highly inbred lines, and none of these lines were lost prior to the learning assays. Taken together, our results indicate a substantial, but not too severe, effect of inbreeding on learning (on average about 18% in the highly inbred lines). The inbreeding depression for learning performance thus appears to be lower than inbreeding depression for viability observed in the same set of lines. It also seems lower than inbreeding depression for other fitness-related traits, such as number of surviving offspring per female (87% of inbreeding depression in competitive conditions, and 27% under uncrowded conditions; Latter *et al.*, 1995), male mating competitive ability (decrease of 5.9–10.7% per 10% increase in F; Sharp, 1984) or aberrant courtship pattern (Miller *et al.*, 1993). One complicating factor in such comparisons is that the observed homozygosity in the inbred lines may have possibly been lower than expected (expected $F = 0.75$–0.93 in our highly inbred lines), because natural selection during the inbreeding process may have favoured heterozygous individuals (Rumball *et al.*, 1994). However, this also applies to other inbreeding studies, so it should not affect the conclusion that learning performance seems less affected by inbreeding than some other fitness-related traits.

Apparently, not all behavioural traits are impaired by inbreeding; in our study, the unconditioned responses to odours did not show inbreeding depression. This indicates that cognitive traits differ in their natural genetic variability and/or in their genetic architecture. This result also suggests that this trait, which is also related to several behaviours based on odour perception, may be under particularly strong purifying selection.

Despite the heavy loss of lines in the course of inbreeding, we found no direct evidence that deleterious alleles have been purged during inbreeding, neither for learning nor for egg-to-adult viability. In our study, crossing inbred lines restored learning performance to the level of, but not beyond, the performance of the outbred flies, and the viability was intermediate between the inbred and outbred flies. Nonetheless, some purging may have still occurred; purging can be difficult to detect, because of a variety of reasons (Ballou, 1997). According to previous studies, only 20% of mammal species tested, and 24% of plants showed purging with very variable ranges (Crnokrak & Barrett, 2002). Moreover, purging may vary substantially even among populations of the same species (Dudash & Carr, 1998; Lacy & Ballou, 1998). It has been shown that purging is more efficient in large populations (Lande & Schemske, 1985; Hedrick, 1994; Wang *et al.*, 1999) and for alleles with large effects (Glémin, 2003). Deleterious alleles with weak effects are difficult to purge, because the effects of genetic drift may outweigh purging selection for these alleles (Lynch *et al.*, 1995; Glémin, 2003). The only partial restoration of viability in the between-line crosses was not because of fertilization failure (eggs showing no signs of development were eliminated from the assays, see Materials and methods). However, the parents in the crosses were themselves inbred, so the incomplete restoration of viability in the crosses may have been because of low quality of offspring produced by inbred mothers. Hence, even though other explanations cannot be excluded, this observation may reflect an effect of maternal inbreeding. If so, such maternal inbreeding effect would mask a potential effect of purging of deleterious alleles.

Learning performance varied significantly among inbred lines, with some lines showing the same learning ability as the outbred and some lines showing clear inbreeding depression. Assuming that all inbred lines had increased homozygosity at genes affecting learning, this suggests that homozygosity only on average, but not in all cases, leads to reduced learning scores. Hence, these results are more consistent with partial dominance rather than overdominance as the main mechanism contributing to inbreeding depression for learning (Charlesworth & Charlesworth, 1999). Furthermore, variation in learning performance among our inbred lines conformed very well to the normal distribution, and even in the worst-performing line the learning score was only reduced by half compared with the outbred flies. This suggests that this variation is caused by multiple loci with small effects on learning ability. It is still possible that some alleles causing major learning impairment were lost in the course of inbreeding with the lines that went extinct.

However, as discussed earlier, the crosses between inbred lines provided no evidence of such purging. Furthermore, the additional set of moderately inbred lines, assayed before any line loss, showed a similar degree of inbreeding depression. Thus, even though mutants unable to learn have been identified in laboratory screens (Dudai *et al.*, 1976; Davis, 2005), such mutants must have been rare or absent in the natural population from which our flies originated. This would indicate that in nature such mutants are strongly selected against, either because strong learning impairment greatly reduces fitness or because such mutants suffer from other deleterious effects.

The fact that on average inbreeding depression for learning is moderate, despite large variation among inbred lines suggests that, in the gene pool of the base population, alleles that reduce learning were not exclusively or predominantly recessive. This is consistent with the notion that, within the normal range of variation, learning ability is under stabilizing rather than directional selection. Under directional selection on a quantitative trait, alleles that reduce the trait value are eliminated more readily if they are dominant rather than recessive. Recessive deleterious alleles are thus more likely maintained and may reach higher frequencies; as a consequence, the standing genetic variation is expected to show directional dominance (Lynch & Walsh, 1998). In contrast, under stabilizing selection on a polygenic trait, alleles that increase the trait value are as likely to be deleterious as those that decrease the trait value. Hence, which polymorphisms are maintained under stabilizing selection is not affected by the direction of dominance (Lynch & Walsh, 1998; DeRose & Roff, 1999), although there may still be some directional dominance for physiological reasons (Wright, 1934).

Selection experiments with *Drosophila* also suggested that learning performance in natural *Drosophila* populations is indeed under stabilizing rather than directional selection (i.e. is optimized rather than maximized). First, learning performance of fruit flies can be readily improved by experimental selection (Lofdahl *et al.*, 1992; Mery & Kawecki, 2002; Reif *et al.*, 2002; Dunlap & Stephens, 2009). Second, some selection experiments demonstrated negative genetic correlations between learning ability and other fitness-related traits, such as larval competitive ability, tolerance to chronic malnutrition or lifespan (Mery & Kawecki, 2003; Burger *et al.*, 2008; Kolss & Kawecki, 2008). The resulting evolutionary trade-offs would constrain the evolution of superior learning performance (Roff & Fairbairn, 2007).

However, in the present study, learning performance was positively correlated across inbred lines with viability. This suggests that some homozygous allele effects reducing viability had negative pleiotropic effects on learning performance. These might, for instance, be because of alleles involved in some general biological functions; impairing these general functions affects a multitude of traits, including cognitive ones. As a result, only healthy flies capable of high survival would be good learners. The positive genetic correlation between viability and learning performance stands in contrast to negative genetic correlations between learning and fitness-related traits observed in selection experiments (Mery & Kawecki, 2003; Kolss & Kawecki, 2008). This apparent contradiction could be in part because of different base populations or different conditions under which viability was assayed [standard food medium here, low food quantity in Mery & Kawecki (2003) and poor food quality in Kolss & Kawecki (2008)]. However, it could also imply that the response to selection and variation among inbred lines are largely based on different sets of loci. The response to selection for better learning is likely to be based on effects of a few, possibly initially rare alleles, which may improve the trait under selection beyond the average of the population, but which may also show antagonistic pleiotropy. Consistent with this notion, line cross-analysis suggests that the response to selection for better learning in Mery & Kawecki's (2002) experiment was based on a few alleles of large effects on learning traits (Kawecki & Mery, 2006). In contrast, as argued earlier, variation among our inbred lines seems to reflect cumulative effects of a larger number of loci, most of which do not specifically affect learning, but rather have broad, positively correlated, effects on various aspects of performance, including viability. Other things equal, loci with even allele frequencies are expected to contribute more to variance among inbred lines than loci with skewed allele frequencies (Crow, 1986). Furthermore, even though additive effects contribute to variation among inbred lines, much of the variation may be because of different numbers of recessive deleterious alleles fixed in different lines. Hence, the positive correlation between learning and viability across inbred lines does not preclude the existence of a trade-off between them.

Only a small number of other studies have investigated inbreeding depression of cognitive functions, most of them finding that these functions are sensitive to inbreeding depression. For instance, spatial learning ability in rats is lower in inbred than in (unrelated) outbred strains (Harker & Whishaw, 2002), and correlative data suggest that inbreeding depression also affects cognitive abilities in humans (Bashi, 1977; Afzal, 1988; Rudan *et al.*, 2002; Abu-Rabia & Maroun, 2005), although not systematically (Neel *et al.*, 1970). Human studies are particularly difficult to interpret because socio-economic factors can bias population comparisons. Our experimental approach allowed us to avoid these problems: we could directly compare inbred lines to their ancestral outbred population and eliminate correlation between the degree of inbreeding and the environment. The results indicate that while inbreeding does on average reduce learning ability, the effects are relatively mild and some highly inbred lines learn as well as their

outbred relatives. This latter result is important in view of the fact that the vast majority of research on mechanisms of learning in *Drosophila* is carried out on highly inbred strains. From an evolutionary perspective, our study is consistent with the hypothesis that in natural *Drosophila* populations learning is under stabilizing selection, with substantial genetic variation segregating in the population. As already demonstrated in selection experiments, this genetic variation would allow those populations to evolve rapidly substantially improved learning performance, should the fitness advantage of learning became greater or the trade-offs less important.

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