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Social experience and pheromonal perception can change male–male interactions in *Drosophila melanogaster*

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Summary

Social interaction with conspecifics can influence the developing brain and behaviour of the exposed animal. This experience can involve the exchange and retention of visual, chemical, acoustic and tactile signals. When several *Drosophila melanogaster* male flies are associated with mated females in the presence of food, they show frequent aggressive interactions. To measure the role of social experience on male–male interaction, two tester males – naïve or exposed to sibling(s) during a variable period of their adult development – were confronted in the absence of female and food. The two males displayed homosexual courtship and aggressive behaviours, the frequency, intensity and directionality of which varied according to their experience. The effect of social experience was greatly enhanced between transgenic males partially defective for pheromonal perception, indicating that male inhibitory pheromones are normally used to repress male–male interaction.

Key words: social experience, homosexual courtship, aggressive behaviour, pheromonal perception, *Drosophila melanogaster.*

Introduction

Social experience can change the development of the nervous system and the behaviour of vertebrates and invertebrates alike (Shors et al., 2001; van Praag et al., 2002; Lledo and Gheusi, 2003; Eliassen et al., 2003; Fahrbach et al., 2003; McRobert et al., 2003; Wommack et al., 2003). Interaction with conspecifics involves multiple sensory signals that need to be retained by individuals who can predict the significance of these stimuli in relation with their reproduction, feeding and survival (Engel and Hoy, 1999; Schaal et al., 2000; Wilson and Stevenson, 2003). Experience-dependent change in social behaviour has been studied in the fruitfly *Drosophila melanogaster*, with a particular emphasis on courtship behaviour (Siwicki and Ladeswki, 2003). Male courtship of a virgin female can decline after conditioning with a mated female. Conversely, young males that are exposed to older mature males during early adulthood show increased heterosexual courtship and mating propensity once they became mature (McRobert and Tompkins, 1988). Conversely, mature males presented during 30 min to young immature males will learn to suppress this misdirected courtship for up to 4 h (Gailey et al., 1982). Early imaginal visual deprivation has been shown to affect mate choice (Hirsch et al., 1995). However, no other sensory modality has been shown to be involved in the modulation of experience-dependent behaviour although several experiments suggest that exposure to pheromonal and acoustic cues during development can change sexual behaviour (Gailey et al., 1982; McRobert and Tompkins, 1988).

Apart from sexual behaviour, *Drosophila* females can change their interactions after encountering other females and show aggressive behaviour (Kamyshev et al., 2002; Nilsen et al., 2004), whereas males can display territorial and aggressive behaviours. The occurrence of these two male behaviours overlap because territoriality often results in aggressive interaction, and both are preferentially induced in the presence of mated females with food (Hoffmann and Cacoyianni, 1990; Chen et al., 2002). Furthermore, during aggressive interactions, males very often display behavioural elements similar to those observed during heterosexual courtship (Hoffmann, 1987; Chen et al., 2002). Territorial behaviour has been shown to change after experience with conspecifics because males held in isolation act more aggressively than males held in groups (Hoffmann, 1990). However, the interpretation of these data was complicated by the presence of multiple flies tested during an extended period of observation. Recently, the measure of aggression was standardized with only two males allowed to fight (Chen et al., 2002).

Here, we tested pairs of transgenic *D. melanogaster* male flies defective for one set of sensory structures potentially involved in pheromonal perception (Xu et al., 2002). In the absence of food and of mated females, we found that the variation of social experience (e.g. exposure to one or several siblings) during adult life affected the nature and strength of the behavioural interaction between two transgenic mature

males. Control males tested in similar conditions showed a much weaker interaction.

Materials and methods

Strains and crosses

D. melanogaster (Meigen) strains were kept in glass vials (150 ml) containing roughly 30 cm^3 standard cornmeal and yeast medium at 25°C under a 12 h:12 h dark:light cycle, with 65±5% humidity. Canton-S (Cs) is a widely used wild-type strain. Dijon 2000 (*Di2*), another wild-type strain, was initiated with five pairs of flies caught during the year 2000 in an orchard at Dijon, France. *CheB42a-Gal4; UAS-GFP* is a transgenic strain homozygous for the two transgenes inserted on chrosomosome 3: *CheB42a-Gal4* contains the promoter of *CheB42a*, a male-specific gene expressed only in few taste sensilla of the tarsa (Xu et al., 2002) fused to the yeast *Gal4* sequence; *UAS-GFP* is the reporter transgene specifically targeted by *Gal4* and used to visualize directly the expression of *CheB42a*. For the sake of clarity, this strain and the *CheB42a-Gal4* transgene are named '*B42*'. Two other strains containing the *CheB42a-Gal4* transgene (but not *UAS-GFP*) inserted in different chromosomal positions were also tested (the three *B42* lines were kindly provided by C. Pikielny). To produce *B42/Di2* (or *B42/Cs*) males, five *B42* virgin females were mated with five *Di2* (or Cs) males, and their F1 male progeny collected. We also tested two other male genotypes: *B42* homozygotes and *B42*×*UAS-grim* (*grim* is a pre-apoptosis gene that deletes *B42-Gal4*-expressing cells; Wing et al., 1998).

Grouping procedure

After a light CO_2 anesthesia, 1–3 h after eclosion, tester male flies were kept either isolated or grouped with varying numbers of same-age siblings in a fresh food vial for a controlled period of time (×2=with one; ×5=with four; ×10=with nine). At the end of the grouping period, which generally took place at 14:00 h, tester males were individually aspirated into a fresh food vial. Males grouped until 5 days old were isolated 1 h before the test. In Figs 1–4, the 'grouping period' is shown by a black-filled bar above the histogram, and the open bar represents the 'isolation period'. To distinguish males, wing clipping – equally distributed for each treatment – was performed with a small pair of iris scissors (#14558-11; FST, Heidelberg, Germany). This operation induced no detectable effect on male behaviour (data not shown).

Behavioural tests

All assays were performed on 5-day-old males, 1–5 h after lights on (between 9:00 h and 13:00 h). In all tests, a male was aspirated into the observation chamber (2.8 cm diameter, 0.5 cm high). 10 min later, a second male (for male–male interaction), or two decapitated objects (for discrimination tests) was (were) introduced. The observation period lasted 600 s. The order in which the two intact males were introduced was randomized. We noted the occurrence and duration of various male behaviours that included sequences very similar to those shown during heterosexual courtship (tapping, wing vibrating, licking and rare attempted copulation, for the two types of tests; O'Dell, 2003) as well as chasing and aggressive behaviours (tussling, lunging; Hoffman, 1987; Chen, 2002) between the two intact males. Aggressive episodes were noted in about 5% of cases.

The total percentage of time that each male spent directing a behaviour (courtship or aggressive) is the behavioural index (BI). However, we did not take into account refusal behaviours like wing flicking and jumps that were often performed by courted intact males. For male–male interactions, we calculated the BI difference as follows: if an isolated 'A' male behaved towards a grouped 'B' male during a total of 120 s (BI=20), and if B acted towards A during 30 s (BI=5), the BI difference (15, in favour of A) is represented under the isolated males, between 10 and 55 (as the second bar from the left shown on Fig. 1A). When both males showed similar BIs, their BI difference was very low (or equal to '0'), and these values made up for the central bar of the histogram. For most pairs represented in the central bars of all figures (N=538), males showed either no BI ('0' for both males=67%), or very low BI ($0 < BI \leqslant 10$; 29%). In only 4% of the cases, at least one of the two males showed a substantial behaviour (BI>10). Males of the control *Di2* and Cs strains generally showed very little physical contact, with the exception of a few brief tapping episode that occurred at the beginning of the observation period. This also explains the high central histogram bar noted for pairs of wild-type males (Fig. 4A,B). In many cases, males jumped away to avoid physical contact.

For the discrimination test, we measured the duration of courtship behaviour directed towards each of the two decapitated sex-objects (BI1/BI2). Decapitation of sex objects allowed us to standardize behavioural observations and to prevent flies producing most of their acoustic signals (Ferveur et al., 1995). Some observations were carried out under a red light (25 W with a Kodak Safe-light filter no. 1) to remove visual stimuli (Boll and Noll, 2002).

Statistics

To test for the directionality of male interactions, the BI of the two series of confronted males were compared using a Mann Whitney test (Statview 4.0). The BIs of the two confronted flies can be considered as independent parameters given that each male can direct or receive a behavioural action, or not interact. The comparison between two experimental sets (each one involving two males) was carried out with a two-factor ANOVA to evaluate the role of social experience, and of the number of siblings or of male genotype. The significance of the probability shown here represents the interaction between these two factors.

For the discrimination experiment, a Student's t-test was used to compare the intensity of behaviour directed towards each sex-object, after testing the normality of the data distribution with an F test.

Results

In a preliminary observation, we found that naive 5-day-old *B42/Di2* male flies carrying one copy of the *B42* transgene (*B42/Di2* males result from the cross between *B42* mothers and *Di2* fathers) showed high levels of homosexual behaviour (CI=38.5±2.3; *N*=94). Males of two other related genotypes also showed high levels of homosexual courtship: *B42* homozygotes (26.4±7.1; *N*=12), and *B42*/UAS-*grim* where *B42* drives the expression of *grim*, a death cell gene (19.3±3.9; *N*=16). We chose to use *B42/Di2* males because (1) of their heterozygous genotype combining the wild-type *Di2* genetic background, and (2) because they showed a stronger male homosexual CI than *B42*/UAS-*grim* (d.f.=119, 2; *F*=6.207; *P*=0.0027). All results shown on Figs 1–3 were obtained with *B42/Di2* males.

The perception of male pheromones is altered in transgenic males

The *B42* transgene contains the promoter of the *CheB42a* male-specific factor, which is secreted by a small number of non-neuronal cells associated with several chemosensory hairs carried by the male frontal legs (Xu et al., 2002). Because *B42* is potentially involved in pheromonal perception, the discrimination of *B42/Di2* males was measured towards two decapitated control flies – a female and a male – that were simultaneously presented for 10 min (Table 1). Discrimination experiments were performed either in red light to remove all visual stimuli or in white light. In red light, *B42/Di2* males could not discriminate the sex of control flies (*P*=0.19), whereas sexual discrimination was possible in white light (*P*=0.015). This contrasts with wild-type subject males, which were able to discriminate sex-object both in red light and in white light (*P*<0.001).

Table 1. *Pheromonal discrimination is affected in* B42/Di2 *males*

	Genotype of tester male			
	B42/Di2		Cs	
Decapitated object fly	Red light	White light	Red light	White light
Cs female	41.3	52.3	43.3	46.8
BI1	(5.5)	(6.0)	(4.4)	(4.2)
Cs male	31	31.6	6.9	7.3
BI2	(5.6)	(5.7)	(1.8)	(1.7)
t	1.31	2.5	7.67	8.68
P	0.19	0.015	<0.001	<0.001

Single tester males of transgenic (*B42/Di2*) and control (Cs) genotype were simultaneously presented to two decapitated objects flies – a female and a male – of the Cs strain, and the duration of their behavioural index was measured towards each object fly (BI1, BI2). The observation, carried out in red light or in white light, lasted 10 min. Data shown are mean BIs (±S.E.M.) for *N*=42–56 tests. The statistical difference between BI1 and BI2 was tested with a Student's *t*-test, and the level of significance is shown below.

Social experience decreases male–male interaction

The aim of this study was to determine whether social experience during adult development can influence behavioural interactions between differently exposed male flies. Social experience with sibling male(s) clearly changed the pattern of interaction between two hybrid male flies of similar genotype (*B42/Di2*) and age (5 days old). The effect of experience is revealed by the comparison of two extreme situations (shown on the top and bottom of Fig. 1). Fig. 1A represents the confrontation between a male kept isolated throughout his adult life (left) and a second male that was held grouped during his adult life with four same-age sibling males (right). The left two bars of the histogram indicate that most isolated males directed an intense behaviour towards grouped males (the reciprocal situation was not observed) during the 10 min observation period. Isolated males frequently showed behavioural sequences similar to those displayed during heterosexual courtship: sustained unilateral wing vibration while chasing, and attempts to lick the genitalia. In response, grouped males often flicked both wings simultaneously and jumped away indicating that they refused the advance of the chaser (Paillette et al., 1991). The central bar of the histogram indicates the absence of a strong directional behaviour, caused by a mutual neutralization, in 27% of the pairs (see Material and methods). The total amount of time spent in directional behaviour (behavioural index = BI, with its mean ± S.E.M. shown above each treatment) indicates the high activity of isolated males (56±6) and the absence of activity in grouped males. In this case, the difference between the BIs of the two males was highly significant (*P*<0.001; see † between treatments above the central bar). By contrast, Fig. 1F represents the confrontation between two males held in isolation during all adult development (from eclosion to test). These naive males showed strong and reciprocal interactions consisting of courtship elements (wing extension and flickering) sometimes combined with sequences characteristic of aggressive behaviour (lunging and tussling; Hoffman, 1987). In this case, the BIs shown by the two males (31±6 and 37±6) were not significantly different (*P*>0.05). No difference of directionality was detected between males and only 19% pairs showed a mutual neutralization.

To outline the period of imaginal development during which social experience with siblings can influence homosexual interaction, tester males were held with siblings for various periods of adult development. The experiments shown on Fig. 1 always involved a male raised in total isolation (left) with a second male held grouped between eclosion and 1–4 days of adult development. Isolated males directed a strong directional BI towards males that were grouped at least 2 days after eclosion. The behavioural directionality and the difference of BIs increased in favour of the isolated male as a function of the duration of grouping.

The number of siblings has no effect on experience

To determine whether the frequency or intensity of social experience can influence male–male interaction, we varied the

number of siblings in the 'conditioning groups' (×2, ×10; Fig. 2A–D) and compared the effect that they induced with that of ×5 groups (Fig. 1). Isolated tester males similarly behaved towards ×2 or ×5 males grouped for 5 days (Fig. 2A and Fig. 1A; d.f.=159; F=0.183; P=0.67). Isolated males also behaved very similarly towards ×10 and ×5 males grouped for 3 days after eclosion (Fig. 2B and Fig. 1C; d.f.=391; F=0.034; P=0.86). The confrontation between ×2 and ×10 males grouped for either 3 or 5 days after eclosion induced weak male–male interaction without directionality (Fig. 2C,D). Instead, the frequency of mutual neutralization increased with the duration of grouping (59% for 3 days; 91% for 5 days).

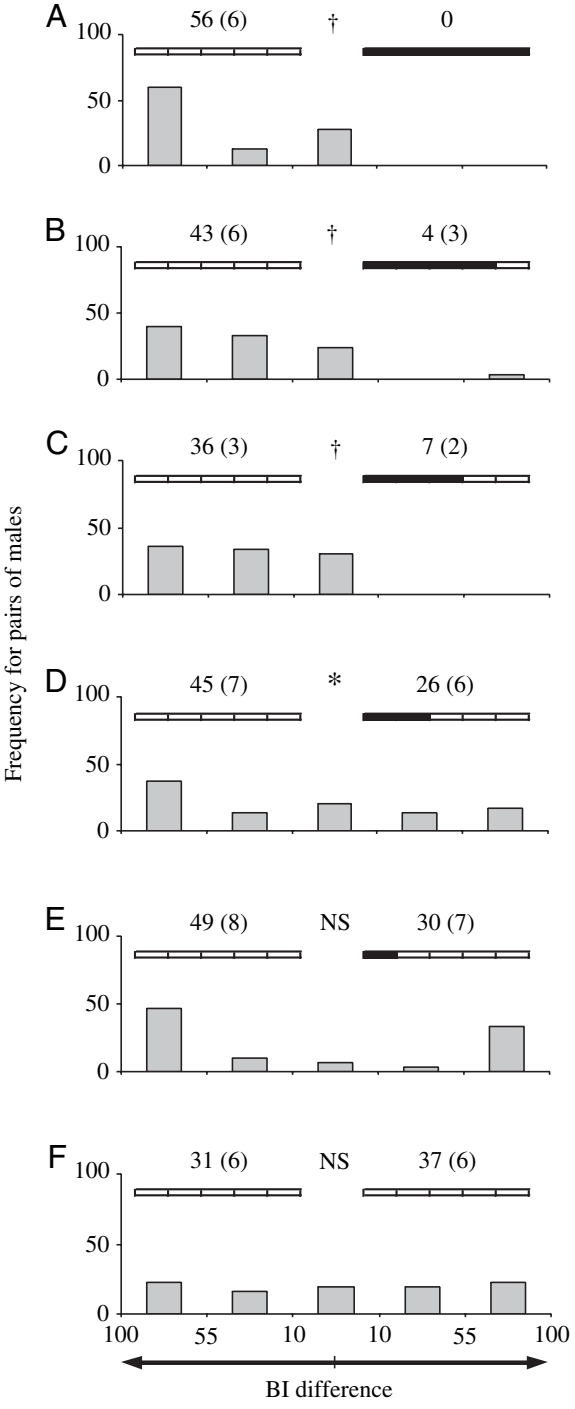

Fig. 1. The strength and directionality of male–male interaction varies with male social experience. In each test, two tester males of similar age (5 days old) and genotype (*B42/Di2*) were paired, and their reciprocal behavioural interaction noted during 10 min. For each test, the duration and nature of social experience of each of the two males is shown above the histograms (the 'experience line', which is shown as a bar). Each experience line is divided into five segments representing the 5 days of adult life (from eclosion to the test); a black-filled bar represents grouping with four other same-age and genotype siblings; an open bar represents the period during which the tester male was kept in isolation. The numbers shown above each experience line indicate the mean of the behavioural index (BI ± S.E.M.) for each treatment, and the statistical significance between treatments is represented above the middle bar ($^{\dagger}P$<0.001; *P<0.05; NS, not significant). The histograms indicate the frequency for pairs of males according to their BI difference: the two bars on the left represent the cases of interactions where the 'left' male directed a higher BI (differences >10 and >55) towards the 'right' male than the reciprocal situation; and *vice versa* for the two bars shown on the right (see also Materials and methods). The middle bar (difference <10) represents the cases with low or no male–male interaction. N=30–54 except in (C) (120).

The effect of experience varies with the exposure period…

The experiment shown in Fig. 2D suggests that two males grouped with siblings until the day of the test very frequently neutralized their mutual interactions. To measure the effect of social exposure during the days preceding the test, one tester male grouped ×5 during all adulthood was confronted with a male grouped ×5 that was isolated either 1, 2 or 3 days before the test (respectively shown on the right and left sides of the histograms of Fig. 2E–G). Social experience induced significant effect during the 2 days preceding the test because 36% males isolated 1 day before the test chased constantly grouped males. This frequency only slightly increased, together with the BIs of the males that were isolated earlier. In these experiments, constantly grouped males rarely chased temporarily grouped males.

…but not with its duration

To distinguish the effect of the isolation period (the lap between isolation and test) from the effect of experience duration, males to be confronted were grouped at different ages but isolated on the same day (Fig. 3A–E). The frequency of mutual neutralization increased when the isolation period decreased: neutralization was very frequent when the isolation period was shorter than 3 days. These data also indicate that the total duration of grouping had no influence on the directionality of male–male interaction. Interestingly, males held during either one or 3 days after eclosion showed low BIs that were not significantly different even if a tendency was noted in favour of males isolated earlier (Fig. 3F).

The effect of experience varies with male genotype

To test the incidence of the *B42* transgene on the intensity and directionality of male–male interaction, various male genotypes were compared. Unlike *B42/Di2* males (Figs 1A, 2A), no or very little interaction was observed between grouped and isolated males of the two wild-type strains Canton-S (Cs) and Dijon2000 (*Di2*; Fig. 4). However, a significant effect (P=0.0018) was detected between isolated and grouped Cs males. When wild-type males were paired, they were generally very active and strongly avoided mutual physical contact with the exception of rare and brief tapping episodes.

B42/Cs males (containing the *B42* transgene in the Cs genetic background) showed a high behavioural directionality between isolated and grouped males. However, the directionality of this interaction was weaker than that shown by *B42/Di2* males raised in the same social conditions (Figs 1A, 4C; d.f.=193; F=9.773; P=0.002). Moreover, the pairing of one naive *B42/Di2* male with a naive *B42/Cs* male induced an interaction that was similar to that observed between two naive *B42/Di2* males (Figs 1F, 4D; d.f.=175; F=2.296; P=0.131). Finally, two independent transgenic lines carrying a single copy of the *B42–Gal4* transgene also induced a strong directional behaviour between isolated and grouped males (data not shown).

Discussion

We found that the nature and strength of the first behavioural interaction between two males depends upon both their genotype and experience. *D. melanogaster* male and female flies have a relatively limited social repertoire that mostly consists of aggressive and sexual interactions (Dow and von Schilcher, 1975; Chen et al., 2002; Nilsen et al., 2004). Environmental parameters can also change social interaction, because inter-male aggressive behaviour is particularly strong in the presence of a mated female and food (Skrzipek et al., 1979; Hoffman, 1987). When two males of similar age and genotypes were paired in a small chamber without mated females nor food, they induced a mixture of homosexual and aggressive behaviours. The proportion and intensity of these behaviours varied according to their mutual social experience: a strong courtship-like behaviour was always directed by the naive male to the experienced male, and aggressive sequences were mostly observed between two naive males.

Among the parameters that contributed to social experience, the most significant was the exposure period during adult development. The highest proportion of mutual neutralization occurred when both tester males were isolated <3 days before the test. When the two males were isolated earlier, their interactions were strong but non-directional. Male–male behaviour was directional only between (1) an isolated male towards a male grouped at least during the 3 days after eclosion, and (2) a male isolated at least 1 day before the test towards a constantly grouped male. However, the total duration

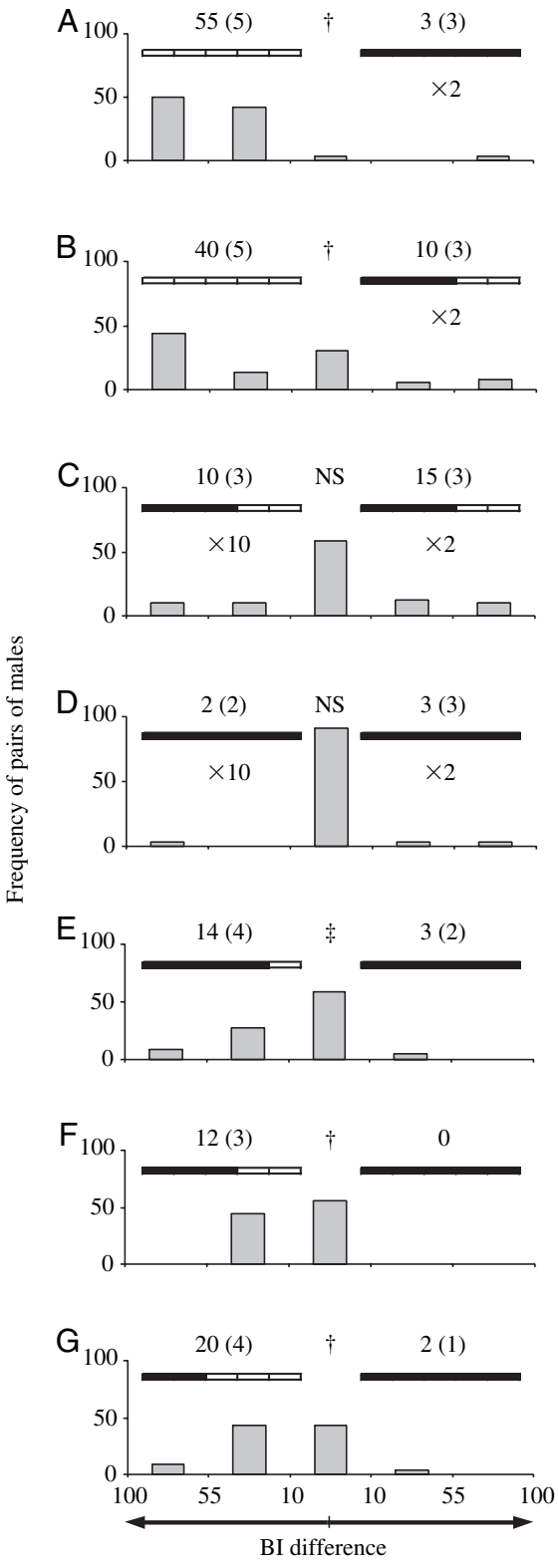

Fig. 2. The effect of social experience is not affected by the number of siblings, but changes with exposure during the days preceding the test. Four same-age siblings were grouped with the tester male during social exposure unless noted ×2 (with one sibling; A–D), or ×10 (with nine siblings; C,D). All tested flies were 5 days old *B42/Di2* males. N=22–35, except B (76), C (73) and F (16). For all other parameters, refer to Fig. 1. ‡P<0.01.

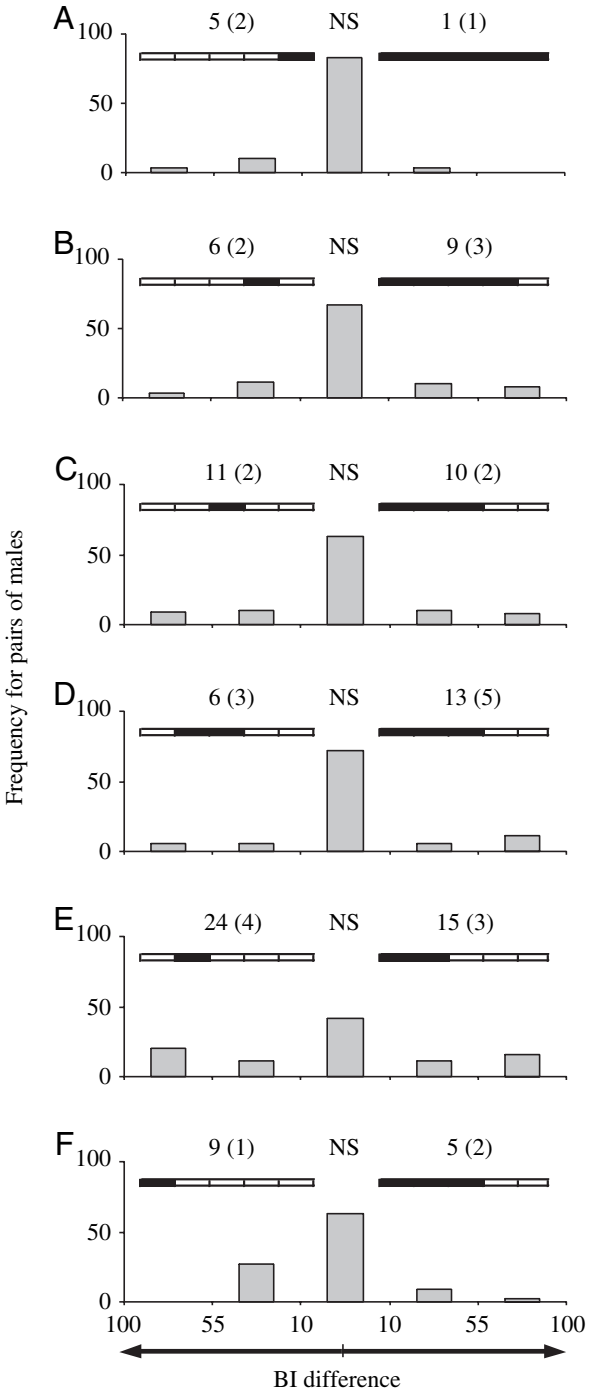

Fig. 3. The isolation period, but not the duration of social exposure, influences the strengh of male–male interaction. All tester males were grouped for different periods of their imaginal development with four other same-age siblings. All flies were 5 days old *B42/Di2* males. *N*=56–82, except C (101), and D (35). For all other parameters, refer to Fig. 1.

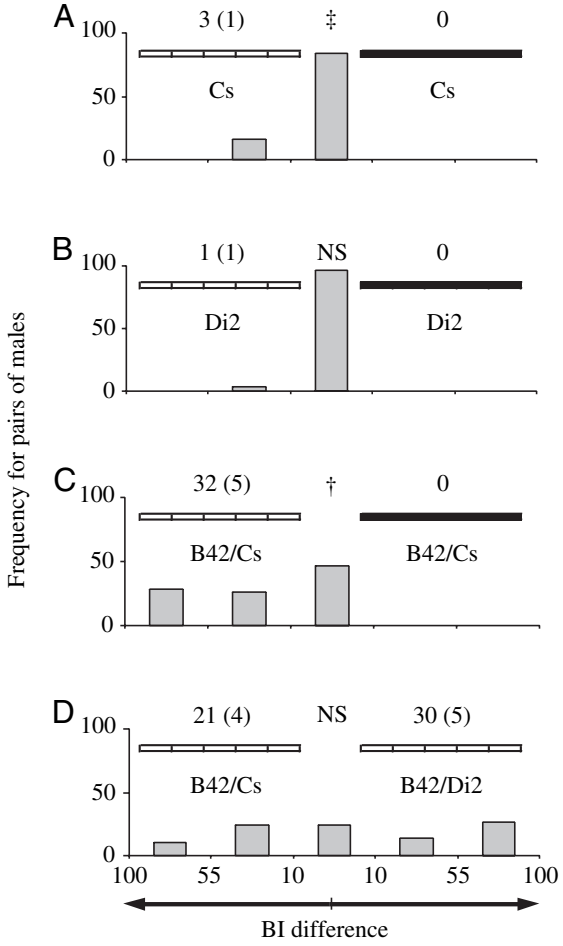

Fig. 4. Male genotype influences the effect of social experience. All tester males were 5 days old, and were from (A) the Canton-S strain (Cs), (B) the Dijon2000 (*Di2*) strain, or (C,D) from the cross between a *B42* female and either a Cs male (*B42/Cs;* C,D left), or a *Di2* male (*B42/Di2;* D, right). *N*=28–57. For all other parameters, refer to Fig. 1. ‡$P<0.01$.

of the social experience and the size of the group (2–10 siblings) had no effect.

The fact that social exposure during different developmental periods induced a strong behavioural directionality (between *B42/Di2* males) suggests that associative and/or non-associative learning processes could be involved. Previous studies revealed that a 30 min exposure period induced an experience-dependent courtship modification (EDCM) that lasted for 4 h (Gailey et al., 1982), whereas a 5 h long associative training induced a change that lasted >9 days (McBride et al., 1999). Given that with our procedure the exposure period lasted more than 24 h and its effect was still detected 3–4 days later, both learning mechanisms, as well as a remodelling of the nervous system, could be involved. We observed that naive *B42/Di2* males strongly and mutually interact during the grouping period (data not shown). It is possible that, after at least 24 h of this conditioning, exposed males became habituated and avoid the sensory signals produced by sibling males. The hypothesis of a reinforced EDCM is consistent with the fact that the frequency of mutual neutralization increased with the age of exposure. However, the habituation process by itself cannot explain why exposed males showed no directional interaction and relatively low BIs, 3–4 days after their isolation (Fig. 3E,F). This relatively long

from-isolation-to-test lapse of time suggests that the quality of male–male interactions could be affected by long-term memory involving associative learning.

Why did social exposure produce a strong effect in males carrying a copy of the *B42* transgene, but not in wild-type males? If the two naive wild-type males confronted in a small observation chamber systematically avoided physical contact, it is probably because they constantly exchanged strong aversive stimuli. The moderate but significant difference detected between the two control strains, and also between *B42/Di2* and *B42/Cs* males, indicates that one or several modifier gene(s) of the background can modulate the effect of experience. We believe that *B42/Di2* males showed much stronger physical interaction probably because of their defective perception of inhibitory male pheromones. This interpretation is supported by the fact that the sensory structures, where *B42* is expressed, are required to detect the pheromones of conspecific flies (Robertson, 1983; Balakireva et al., 1998; Xu et al., 2002; Bray and Amrein, 2003). The altered behaviour of *B42/Di2* males could result from the toxic effect of Gal4, or GFP, or of both products, in the *B42–Gal4*-expressing cells, causing a similar effect to the *grim* gene that killed the same cells. If it is true, it means that the *B42* transgene affects a function of the targeted subset of tarsal hairs that normally allow male flies to detect inhibitory pheromones of conspecific males (Ferveur and Sureau, 1996). Alternatively, could the interaction between males be the result of their pheromonal difference after the passive transfer of cuticular hydrocarbons during conditioning? This hypothesis can be ruled out because (1) most hydrocarbons transferred by rub-off are eliminated from the cuticle after few hours (Scott and Jackson, 1990), and (2) males that were either kept with one or with nine siblings induced no behavioural difference. Therefore, if social experience can theoretically affect all males, its effect is better measured in males with defective sensory perception.

Male inhibitory pheromones, which strongly prevent homosexual interaction between wild-type males, can lead to intense fighting episodes in the presence of mated females and food (Hoffman, 1987; Chen et al., 2002). This shows that male inhibitory pheromones act in a context-dependent manner, and we hypothesize that when these substances are perceived simultaneously with chemical signals inducing rewarding effects (female pheromone, food odors), they can elicit male–male antagonistic behaviours. Although it remains to be shown that sensory integration of different pheromonal inputs can change the release of substance(s) that affect neural function, certain neuromodulators present in the central nervous system can precisely control male courtship and aggressive behaviours (Yellman et al., 1997; Neckameyer, 1998; Lee and Hall, 2001; Baier et al., 2002). We note that the experimental manipulation of β-alanine changes male aggressivity (Jacobs, 1978), and this effect could be partly caused by male defective visual acuity (Baier et al., 2002). This makes an interesting analogy with our data because both observations suggest that the behavioural interaction can be changed between males that are defective for either modality of sensory perception.

In conclusion, the strong directional interaction observed between two *B42* transgenic males – a naive male chasing an experienced male – may result from their very different experience: the naive male courts because he is not (very) inhibited by the pheromones of the exposed male who escapes because he retains a negative experience of the repulsive signals produced by sibling males.

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