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Is mate choice in *Drosophila* males guided by olfactory or gustatory pheromones?

Claude Everaerts *, Fabien Lacaille, Jean-François Ferveur  

Unité Mixte de Recherche CSGA (Centre des Sciences du Goût et de l’Alimentation) associée au CNRS, Université de Bourgogne, Faculté des Sciences  

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*Correspondence: C. Everaerts, Unité Mixte de Recherche CSGA (Centre des Sciences du Goût et de l’Alimentation) associée au CNRS, Université de Bourgogne, Faculté des Sciences, 6, Bd Gabriel, 21 000 Dijon, France.  
E-mail address: Claude.Everaerts@u-bourgogne.fr (C. Everaerts).

**Key words:** courtship; cuticular hydrocarbon; discrimination; *Drosophila melanogaster*; mate choice; olfaction ; sex pheromone; taste
*Drosophila melanogaster* flies use both olfactory and taste systems to detect sex pheromones and select the most suitable mate for reproduction. In nature, flies often face multiple potential partners and should have an acute sensory ability to discriminate between different pheromonal bouquets. We investigated both the pheromones and the chemosensory neurons influencing *Drosophila* mate choice. We measured various courtship traits in single tester males simultaneously presented with two target male and/or female flies carrying different pheromonal bouquets (pairs of control flies of the same or different sex, same-sex target pairs of pheromonal variant strains). The courtship traits reflected the perception of either olfactory cues perceived before or gustatory cues perceived after the first physical taste contact. Our results suggest that male mate choice exists in *D. melanogaster* and that male discrimination between potential mates could be a two-step process involving chemical cues perceived before and after the first gustatory contact. In addition, when a male was simultaneously presented with two potential sexual partners, the olfactory and gustatory cues he used depended on the pheromonal patterns of both flies, but his response could also depend on additional effects resulting from the simultaneous perception of the two flies, leading to a nonlinear choice of a sexual partner. Moreover, some tester males with genetically altered gustatory receptor neurons strongly changed their partner preference, indicating that the fly’s peripheral nervous system is essential for pheromonal detection and mate choice.
For animals, it is crucial to select the fittest mating partner to produce progeny and the ‘grossest blunder’ in sexual behaviour would be to mate with a partner that does not ensure the greatest chance of producing the most viable offspring (Fisher 1930, page 130). In higher animals, both sexes are adapted to avoid this blunder and their nervous system is built in such a way that mating with an unsuitable partner is usually inhibited by differences in the ‘appearance or behaviour’ of this individual (Fisher, page 130). However, these signals could instead indicate the high genetic quality of the bearer and constitute ‘viability indicators’ (Andersson 1994; Kokko 2001). In many animals, chemical signals are often used as viability indicators detected by smell and taste which allow individuals to select potential mates (Wyatt 2003).

In *Drosophila*, as in many dipterans, most known sex pheromones are cuticular hydrocarbons (CHs; Wicker-Thomas 2007) which are detected by the olfactory and/or gustatory sensory systems. As in other insects, olfactory and gustatory receptors in *Drosophila* are found in neurons housed in various sensory appendages (Dethier 1976). These neurons can perceive chemical stimuli, transduce them and convey the corresponding information to the central nervous system, which in turn will trigger the appropriate behavioural response (Wang et al. 2004).

In *Drosophila melanogaster*, gustatory receptors and gustatory receptor neurons (GRNs) are relatively well characterized (Hiroi et al. 2004; Thorne et al. 2004; Marella et al. 2006; Moon et al. 2006; Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Kent & Robertson 2009). More specifically, a small group of GRNs present on the labial palps and legs carrying the Gr66a receptor are involved in the taste detection of both bitter substances and 7-tricosene (7-T), a male CH which acts as a sex pheromone inhibiting male and stimulating female flies (Ferveur & Sureau 1996; Grillet et al. 2006; Moon et al. 2006; Lacaille et al. 2007, 2009). Both types of compound induce a similar aversive dose-dependent effect on male courtship
and related feeding behaviours (Lacaille et al. 2007, 2009). We hypothesized that the Gr66a-expressing taste neurons were initially used to detect and respond to toxic food, and subsequently co-opted into the new function of detecting pheromonal stimuli carried by other males to avoid homosexual interactions (Lacaille et al. 2007). This hypothesis does not exclude the possibility that other groups of taste neurons and/or sensory modalities are also involved in the male avoidance response. We also found that while males with altered GR66a neurons showed no, or very little, avoidance (Lacaille et al. 2007), the genetic feminization of the same neurons (in otherwise male flies) enhanced their avoidance response to 7-T and bitter molecules (Lacaille et al. 2009).

In the field, Drosophila adults of different species and strains aggregate on food patches where courtship, mating and oviposition occur (Wertheim et al. 2005, 2006). Aggregation behaviour is influenced by food sensory stimuli, social interaction and individual experience (Tinette et al. 2004). As they live in heterotypic groups, Drosophila flies must have an accurate sensory system to discriminate the appropriate partner from all potential ones. This process of discrimination probably involves multiple sensory signals.

The genetic dissection of D. melanogaster courtship behaviour has allowed researchers to unravel the mechanisms of interindividual communication involving species- and sex-specific sensory signals (Greenspan & Ferveur 2000; Hall 2002). Visual, olfactory, gustatory, acoustic and/or tactile stimuli are exchanged by pairs of mature D. melanogaster flies (reviewed in Greenspan & Ferveur 2000) and the degree of specificity of these signals could increase as courtship progresses (Arienti 1993; Cobb & Ferveur 1996). Since it was first described by Sturtevant (1915), most studies of D. melanogaster courtship have exclusively focused on male behaviour while female precopulatory behaviour has often been assigned the role of an ‘accept - or - reject’ switch leading to mating. However, (Lasbleiz et al. 2006) showed that the female constantly interacts with the male during courtship and is important for mating
success. This is consistent with the costs of reproduction to *Drosophila* females (Ikeda 1974; Turner & Anderson 1983; Chapman et al. 1995; Sgro & Partridge 1999; Chapman 2001; Lung et al. 2002). However, reproductive costs also reduce life span and future fertility in male *D. melanogaster* (Cordts & Partridge 1996). Although male mate choice remains poorly documented, available evidence suggests that it is widespread among insects and other animals (Bonduriansky 2001; Kraaijeveld et al. 2007). The most commonly observed male mate choice is precopulatory mate choice which tends to maximize a male's expected offspring from each mating by favouring female phenotypes associated with high fecundity or reduced sperm competition intensity (Bonduriansky 2001). Male insects typically choose females based on easily detectable phenotypic indicators of fecundity (Cordts & Partridge 1996), possibly including volatile and contact chemical cues.

In *D. melanogaster*, sex pheromones are CHs with little or no volatility (Ferveur 2005; Wicker-Thomas 2007) and are therefore mainly perceived by gustation (Stocker 1994; Shanbhag et al. 2001; Boll & Noll 2002; Lacaille et al. 2007) but sometimes also by short-distance olfaction (Ferveur 2005; Grillet et al. 2006; Benton et al. 2009). Therefore, the choice of a mate by a *Drosophila* male could depend on both smell and taste. In the present study, we addressed three questions: (1) do male flies use CHs to choose between two partners simultaneously present, (2) do they use volatile or nonvolatile cues and (3) are Gr66a neurons involved in this process?

**METHODS**

**Fly husbandry**

All *D. melanogaster* strains were raised and tested at 24 ± 0.5 °C and 65 ± 5% relative humidity on a 12:12 h light:dark cycle. Stocks were maintained on alcohol-free medium
mixed with killed yeast in 150 ml glass vials. When 1-2 h old, flies were sexed under light carbon dioxide anaesthesia at 2 - 4 h after lights on, and were kept in fresh-food vials either isolated (males) or in groups of five (females) until the age of 4 days.

**Genetics**

**Target flies**

The targets consisted of mature male and female flies from two wild-type strains, Canton-S (CS) and Tai and from one mutant strain, desat1. CS and Tai strains had been maintained in the laboratory for several decades, and the desat1 strain for more than one decade.

In these three strains, both sexes strongly diverge in their cuticular hydrocarbon production. CS is a wild-type strain (caught in the U.S.A.) used in many laboratories and representative of cosmopolitan *D. melanogaster* strains: males produce high levels of 7-tricosene (7-T) and low levels of 7-pentacosene (7-P) whereas females predominantly produce 7,11-dienes (7,11-hepta- and nonacosadiene: 7,11-HD, 7,11-ND; Antony & Jallon 1982; Jallon 1984). Tai is a variant strain (caught in the Ivory Coast) where males show low 7-T and high 7-P levels whereas females produce much higher levels of 5,9-hepta- and nonacosadiene (5,9-HD, 5,9-ND) than of 7,11-dienes (Jallon 1984; Jallon & Pechine 1989; Savarit & Ferveur 2002). Flies of the desat1 mutant strains almost completely lack unsaturated cuticular hydrocarbons (monoenes and dienes) but produce high levels of cuticular alkanes with 23 and 25 carbons (23-Lin, 25-Lin; Marcillac et al. 2005).

**Tester males**

Tester males (whose behaviour was measured) belonged to the CS strain or were genetically manipulated for their Gr66a-Gal4-expressing neurons (Lacaille et al. 2009): 66a-Gal4/+ and 66a-Gal4/UAS-tra males.
The Gr66a transgenic males carry, on chromosome III, a single copy of the Gr66a-Gal4 transgene which contains a promoter of the gustatory receptor Gr66a gene fused with the yeast Gal4 sequence (Dunipace et al. 2001). We used the Gr66a-Gal4 transgene to target and activate the UAS-transformer (UAS-tra; Ferveur et al. 1995) reporter transgene which combines the upstream activation sequence (UAS, specifically activated by Gal4; Brand & Perrimon 1993) fused with the cDNA of the transformer sex determination gene, which allowed us to feminize autonomously the Gal4-expressing neurons (see Lacaille et al. 2009).

**<H2>Behaviour**

**<H3>Behavioural tests**

Each single tester male fly was given a choice of two target flies. We measured several behavioural parameters reflecting the male’s ability to perceive both volatile and contact pheromones. These parameters included the courtship latency, the first target chosen, the total duration of courtship (or courtship index, CI) directed towards each target (i.e. the proportion of time that the courting male spends vibrating one wing, curving his abdomen, licking the female genitalia or attempting to copulate; no qualitative difference was noted between the courtship sequences of the different courting males), and the discrimination index (DI) which was adapted from the Malogolowkin - Cohen index (Malogolowkin-Cohen et al. 1965) and computed as follow: DI\textsubscript{xy} = (CI_x - CI_y)/(CI_x + CI_y), where CI\textsubscript{x} and CI\textsubscript{y} are CIs towards the ‘x’ or the ‘y’ target. Besides providing a comparison of the attractiveness of both targets in each pair, the DI alone is insufficient to describe precisely the discrimination ability of tester males. Therefore we also recorded the frequency of males courting each target, and the number of transitions between targets, and we designed two parameters to allow us to characterize both the direction and the intensity of the discrimination, independently of the chosen target. The global choice was computed by classifying the tester males into three
groups depending on whether they did not prefer either target, they courted the x target more or the y target more. The global choice intensity was quantified using the absolute values of the DI. Together, the last three parameters provide a good indicator of the ‘robustness’ of the discrimination. Courtship latency and first target chosen are relevant to chemoperception prior to the first gustatory contact whereas the other parameters provide a measure of the tester male behaviour after his first gustatory contact.

All behavioural tests took place 1 - 4 h after lights on. When tests simultaneously involved male and female targets, they were carried out under a dim red light (25 W with a Kodak Safe-light filter n°1) to remove all sexually dimorphic visual stimuli (Boll & Noll 2002). Tests with same-sex targets were carried out under a white light, so the flies could also see the targets, but, as the two targets were similar, visual cues should theoretically not have influenced the discrimination process. In each pair, a notch made with a microscissor in one wing of either target allowed us to distinguish genotypes. To eliminate behavioural feedback and to reduce the effect of acoustic signals (Ferveur et al. 1995), all target flies were decapitated 30 min before the test under carbon dioxide anaesthesia.

Four-day-old tester males were individually aspirated (without anaesthesia) under a watch glass used as an observation chamber (1.6 cm³). After a 5 min acclimation period, a pair of 4-day-old decapitated target flies was introduced and the CI that the tester male directed towards each of them was measured for 10 min. After each observation, the watch glasses were cleaned using detergent, then pentane and finally purified water. For each behavioural test, $N = 28 - 32$. 
We conducted three types of test. First and to assess the validity of our discrimination parameters, we carried out a control experiment in which we put a single CS tester male with a pair of same-sex CS flies. We also measured the ability of tester males of three genotypes (CS, 66a-Gal4/+ and 66a-Gal4/UAS-tra) to discriminate the sex pheromones of control flies. The behaviour that they directed towards a decapitated target male and a decapitated target female from a wild-type strain (CS) was measured under red light. Second, to assess the ability of the three tester males to discriminate the quality of male cuticular pheromones, we put them with target males of the three variant strains CS, Tai (T) and desat1 (D) with different predominant pheromones. Target females were paired in the three possible combinations and we measured the ability of the three tester males (CS, 66a-Gal4/+ and 66a-Gal4/UAS-tra) to discriminate each genotype within these pairs. Third, we put tester males with target females from CS, Tai (T) and desat1 (D) strains, which also have different predominant cuticular compounds.

Statistical analysis of behavioural data:

Except for percentages (first target chosen, frequency of males courting each target and global choice intensity), all other parameters are expressed as their mean ± SEM. Several statistical treatments were used according to the parameter analysed. The courtship latency, CI, DI, number of transitions between targets and the global choice intensity were tested with Kruskal - Wallis analysis of variance of ranks completed by Dunn's multiple pairwise comparisons (two tailed with Bonferroni correction). The significance of the first target chosen was tested with a binomial test and that of the courting males’ frequency with a z test. The differences in global choice between the tester genotypes were tested with a chi-square test (with a computation of significance by cell) and differences in global choice intensity with a Mann –
Whitney U test. All statistical analyses were conducted with XLSTAT 2007 (Addinsof, New York, U.S.A.).

**Chemical analysis**

**Extraction and gas chromatography analysis:**

Cuticular hydrocarbons (CHs) from 4-day-old individuals from the different tester and target flies were analysed by gas chromatography following hexane extraction and adding synthetic C26 and C31 hydrocarbon internal markers, according to standard procedures (Ferveur 1991). Analyses were performed with a Varian CP3380 chromatograph, fitted with a flame-ionization detector, with a CP-sil/5CB capillary column (Varian, 25 m x 0.32 mm internal diameter) and with a split-splitless injection system (operating with a split flow of 60 ml/min and a septum purge of 3 ml/min, opening of the split port 30 s after injection). Hydrogen was used as carrier gas (50 cm/s velocity at room temperature). The injector and detector temperatures were 260 and 280 °C, respectively. The column was held isothermally at 140 °C for 2 min, then programmed to increase at a rate of 5 °C/min to 280 °C. The data were automatically computed and recorded using PC software (Star 5.2, Varian Inc., Palo Alto, CA, U.S.A.).

**Statistical analysis of target cuticular profiles:**

For each series of target pairs (pairs formed either by CS wild-type flies of the two sexes or by heterotypic flies of the same sex), the absolute amounts of CHs were used to compute an agglomerative hierarchical clustering (AHC; using the Pearson correlation coefficient as a similarity index and the unweighted pair-group average linkage method, at a level of 0.95) allowing us to exclude correlated CHs from further analysis. In each group of correlated compounds we retained the most abundant CH as representative of this cluster.
For each type of target pair, we conducted a discriminant Analysis (DA) using the absolute amounts of the uncorrelated CHs as quantitative variables and the sex or the strain (depending on the target pair) as a qualitative variable. For each type of target pair, we also conducted a forward stepwise DA of the quantitative variables (with an entry threshold value of $P = 0.05$) which led to a 100% correct classification of the individuals. The results of these different DAs were summarized by their confusion matrices after cross-validation, and by the selected CHs for the forward stepwise DA. The AHCs and DAs were computed using XLSTAT 2007.

**RESULTS**

**Discrimination of sex pheromone**

In the control test, no significant difference was found with either male or female pairs, supporting the validity of our parameters (Fig. 1). Moreover, tester CS males directed a much stronger courtship towards females than towards males ($H_2 = 36.09$, $P < 0.0001$; Fig. 1c).

Also, both the frequency of courting males ($z = 4.85$, $P < 0.0001$; Fig. 1e), and the global choice intensity were higher with target females than target males ($U = 225.50$, $P < 0.0001$; Fig. 1h). Examination of the global choice parameter shows that 100% of CS tester males courted target females, while only 37% of tester males courted target males ($\chi^2 = 29.23$, $P < 0.0001$; Fig. 1g).

Under red light, the time taken to initiate courtship (courtship latency; Fig. 2a) varied significantly according to the sex of the target flies and the genotype of the tester male ($H_5 = 10.98$, $P = 0.05$). Both CS and $66a$-Gal4/UAS-tra tester males courted the target female faster than the target male ($H_2 = 16.25$, $P < 0.0001$; Fig. 2a) whereas $66a$-Gal4/+ tester males showed no difference in courtship latency. This observation is consistent with the finding that most CS and $66a$-Gal4/UAS-tra males first courted the target female (binomial
test: CS: $P = 0.001$; 66a-Gal4/UAS-tra: $P < 0.0001$; Fig. 2b), in contrast to 66a-Gal4/+ tester males which showed no significant first choice ($P = 0.570$).

After the first contact, 66a-Gal4/+ tester males showed a lower heterosexual CI, and a higher homosexual CI than CS and 66a-Gal4/UAS-tra tester males ($H^2 = 101.55$, $P < 0.0001$; Fig. 2c). Consequently, the DI of the 66a-Gal4/+ males was much lower than those of the two latter males ($H^2 = 23.29$, $P < 0.0001$; Fig. 2d). However, the different ability to discriminate and to court flies of both sexes did not change the total time that males spent courting (72 - 81% of the total duration; $H^2 = 5.34$, $P = 0.069$).

The comparison of the courting males’ frequency towards each target fly (Fig. 2e) also allowed us to evaluate the intensity of discrimination. While more than 91% of males of the three genotypes courted the target female, the difference in frequency between heterosexual and homosexual courtship varied among tester male genotypes: it was not significant for 66a-Gal4/+ males ($z = 1.08$, $P = 0.281$) while it was highly significant for both CS ($z = 2.98$, $P = 0.0001$) and 66a-Gal4/UAS-tra males ($z = 5.60$, $P < 0.0001$).

The variation in the number of transitions shown by the three tester males confirmed these differences: 66a-Gal4/+ tester males showed more transitions than both CS and 66a-Gal4/UAS-tra males ($H^2 = 14.29$, $P = 0.001$; Fig. 2f). The higher stability showed an inverse relationship with the CI differences. Finally, the global choice parameters (Fig. 2g, h) reflect the general variation in the behavioural parameters. Whereas both CS and 66a-Gal4/UAS-tra testers showed a clear and somewhat equivalent heterosexual preference, the choice shown by 66a-Gal4/+ testers was not so marked. This differed significantly from the choice made by the other two tester types ($\chi^2_2 = 10.90$, $P = 0.028$): the 66a-Gal4/+ tester males chose the female target less often (and consequently the male target more often) than the other two tester males. Similarly, the 66a-Gal4/UAS-tra tester males showed the highest intensity of
global choice whereas the 66a-Gal4/+ males showed the lowest ($H_2 = 22.61, P < 0.0001$; Fig. 1h).

**H2** Discrimination of male pheromones

In the test with male targets, the parameters reflecting the male behaviour before the first physical contact (courtship latency - $H_{17} = 55.17, P < 0.001$ - and first choice; Fig. 3a, b) differed according to both the tester and the target males. With [T | CS] pairs, Tai males always induced a faster courtship than CS males (Fig. 3a) and were the first chosen target (binomial test: $P < 0.004$; Fig. 3b). With [T | D] pairs, the preference for the Tai target male was only significant for CS and 66a-Gal4/+ tester males (binomial test: CS: $P = 0.006$; 66a-Gal4/+: $P = 0.025$; Fig. 3a, b). With [CS | D] pairs, the 66a-Gal4/+ tester males slightly preferred CS target males, whereas CS and 66a-Gal4/UAS-tra testers slightly preferred desat1 target males. However the first choice was only significant for 66a-Gal4/UAS-tra testers (binomial test: $P = 0.039$; Fig. 3b).

After the first contact, Tai target males induced higher CIs (Fig. 3c), resulting in higher DIs (Fig. 3d), except for 66a-Gal4/UAS-tra testers with [T | D] targets. In this case, Tai and desat1 males induced similar CIs resulting in a low discrimination of the [T | D] pair. A similar pattern was found for the frequency of courting males (Fig. 3e). The summed CI towards both targets was high when a Tai target was present ($H_8 = 112.47, P < 0.0001$), and low in the case of [CS | D] targets.

With these [CS | D] targets, 66a-Gal4/UAS-tra testers showed fewer transitions ($H_8 = 16.81, P = 0.032$; Fig. 3f) than in any other situation, indicating that their courtship was very stable although not very high. Conversely, the most transitions were found in 66a-Gal4/UAS-tra testers with [T | D] targets. Finally, the global choice parameters were consistent with the previous parameters: (1) Tai target males were always preferred except by 66a-Gal4/UAS-tra
testers associated with the [T | D] targets and (2) no strong preference was shown with [CS | D] targets.

**Discrimination of female pheromones**

With female targets, male behaviour before physical contact (courtship latency - \( H_{17} = 29.79 \), \( P < 0.028 \) – and first choice; Fig. 4a, b) differed with the combination of target females used. In [CS | D] pairs, CS females always induced a faster courtship than desat1 female (Fig. 4a) and were more often chosen first (binomial test: \( P < 0.03 \); Fig. 4b). With [T | CS] pairs, only 66a-Gal4/+ testers preferred CS females (\( P < 0.001 \); binomial test: \( P = 0.0002 \); Fig. 4a, b), whereas 66a-Gal4/UAS-tra testers preferred Tai females and CS testers showed no preference (binomial test: \( P = 0.05 \); Fig. 4b). These differences are reflected by the difference in courtship latencies (Fig. 4a). With [T | D] pairs, CS and 66a-Gal4/+ testers slightly preferred the Tai target female whereas 66a-Gal4/UAS-tra testers slightly preferred the desat1 target. The CIs of the three tester males were similar with [CS | D] pairs: CS females always induced a higher CI than desat1 females (Fig. 4c). With [T | D] pairs, CS and 66a-Gal4/+ testers slightly preferred Tai females whereas 66a-Gal4/UAS-tra testers preferred desat1 females. With [T | CS] pairs, CS and 66a-Gal4/+ testers courted CS females more intensively than Tai females whereas 66a-Gal4/UAS-tra testers courted the Tai target more intensively. The total amount of courtship did not vary between the testers with the [CS | D] pairs, and was similar with both [T | D] and [T | CS] pairs: CS < 66a-Gal4/+ < Gr66a-Gal4/UAS-tra (\( H_8 = 20.88 \), \( P = 0.007 \)). Independently of tester genotypes, the CS female target was courted more often than the desat1 female in the [CS | D] pair (\( z \) test: \( z = 2.419, 4.536 & 4.526, P < 0.02 \); Fig. 4e). In the [T | CS] pairs, the CS target was also courted more than the Tai female by the 66a-Gal4/+
male (z test: $z: 5.477; P < 0.0001$), while it was courted less by the 66a-Gal4/UAS-tra males (z test: $z: 2.739; P = 0.006$).

The number of transitions differed significantly ($H_8 = 66.26, P < 0.0001$; Fig. 4f). While CS and 66a-Gal4/UAS-tra testers showed a high number of transitions with the [CS | D] pairs, 66a-Gal4/+ testers showed few transitions with [CS | D] pairs. Again the global choice parameter reflected well the situation described above: it showed no variation with the [CS | D] female pairs (the CS target was always preferred), and varied significantly with the [T | D] and the [T | CS] pairs ([T | D]: $\chi^2_2 = 18.86, P = 0.001$; [T | CS]: $\chi^2_2 = 35.26, P < 0.0001$; Fig. 4g). The most obvious differences in global choice were (1) the preference of the 66a-Gal4/UAS-tra tester males for the desat1 female in the [T | D] pairs and for the Tai target in the [T | CS] pairs, and (2) the preference of the 66a-Gal4/+ tester males for the CS female in the [T | CS] pairs. All the global choice intensities showed a similar level, except for both the CS testers with the [CS | D] pairs and 66a-Gal4/+ testers with the [T | D] pairs which showed a lower robustness of global choice ($H_8 = 52.78, P < 0.0001$; Fig. 4h).

The D. melanogaster cuticular profile consists of 58 compounds including the classical monoenes (9-, 7-, 5-tricosenes; 9-, 7-, 5-pentacosenes; 7-heptacosene; 7-nonacosene), dienes (7,11-hepta- and 7, 11-nonacosadiene), linear (L) and methyl branched alkanes (Br) previously described (Everaerts et al., in press). Although the cuticular hydrocarbon profiles were very similar in the three genotypes of tester males (data not shown), the target flies’ CHs were very different. For the sake of clarity, we only show the absolute quantities for the six principal cuticular compounds with a putative pheromonal role in D. melanogaster, namely 7- and 5-tricosene (7-T, 5-T), 9-, 7-pentacosene (9-P, 7-P), and 7,11-hepta- and 7,11-nonacosadiene (7,11-HD and 7,11-ND; Table 1). For each combination of target flies, the
results of the different DAs are shown with their confusion matrices after cross-validation, and with the CHs selected by the forward stepwise DAs (Table 2).

The cuticular profile of the male/female CS targets allowed us to classify correctly 100% of male and female individuals. However, among all CHs the forward stepwise DA indicated that only five were informative enough to obtain this result, namely the $7-T > 5-P > 27-Br > 7,11$-ND $> 7-N$ (CHs are classified according to their contribution to the discriminatory power of our model).

With the chemical data obtained with heterotypic target pairs, the DAs reached a 13.3 - 86.7% correct classification of individuals. Depending upon the combination pair, the forward stepwise DAs indicates that three or four CHs are sufficient to obtain a 100% correct classification.

The compound $7-T$ contributed strongly to the discrimination between target males of the [CS | D] and [T | CS] pairs. Moreover 6-docosene (6-D) and a branched heptacosene (Br-H) could be involved in the [CS | D] pair whereas 9- and 5-P could be used in the [T | CS] pair. On the other hand, $7-T$ seemed not to be used for discrimination in the [T | D] male pair: Tai and desat1 males could be discriminated by 25-Br, 5-P and Br-H.

For female targets, the sets of selected CHs varied more according to the pairs, but they never included dienes. The DA discriminatory power was very low with the [T | D] pair of target females.

**DISCUSSION**

**Male ability to use pheromones to discriminate potential mates**
In the present study, we did not observe any significant difference between the discrimination or the courtship of the CS testers towards the two targets of a same-sex wild-type pair (Fig. 1). However, and as expected, the female targets elicited a stronger and steadier courtship than male targets. Also, CS males could discriminate at a short distance the male and female targets within heterosexual CS pairs (Fig. 2). After the first gustatory contact, the CS tester males courted the female more intensively and more steadily than the male target. Heterotypic pairs of males triggered varied discrimination patterns in CS tester males. Before the first gustatory contact, they first courted the Tai target (within both [T | D] and [T | CS] pairs), while they showed no preference within the [CS | D] pair. The response shown by CS testers after the first gustatory contact is globally consistent with their pregustatory choice: they courted the Tai male targets more within the [T | D] and the [T | CS] pairs, whereas within [CS | D] pairs, they courted the desat-1 male target more intensively. Heterotypic pairs of female targets also triggered a variable pattern of response in wild-type tester males. While the CS tester oriented first to the CS female within the [CS | D] female pairs, no preference was shown with the [T | CS] and [T | D] female pairs. However, after the first gustatory contact, the CS testers courted the CS female targets more intensively within the [CS | D] and [T | CS] female pairs, and the Tai female targets more intensively within the [T | D] female pairs.

In insects, sex chemical communication often relies on both volatile and contact pheromones (Wyatt 2003). In D. melanogaster, most known sex pheromones are CHs with little or no volatility (Ferveur 2005; Wicker-Thomas 2007). These compounds are mainly perceived with the gustatory organs located on the fore tarsi and mouthparts (Stocker 1994; Shanbhag et al. 2001; Boll & Noll 2002; Lacaille et al. 2007). However, some of the lightest cuticular compounds may be volatile enough to be detected at a short distance by the olfactory organs on the head (antennae and maxillary palps; Ferveur 2005; Grillet et al. 2006; Benton et al.)
Therefore, the choice of a sexual partner by a *Drosophila* male could depend successively on smell and taste. First, and at a short distance, olfactory perception could allow a fly to discriminate some chemical features of a potential sex partner. Then after the first gustatory contact, the pheromones perceived with taste appendages could modulate (by either enhancing or inhibiting) male courtship ardour.

To deal with the hypothesis of such a two-step discrimination process, we used two series of ‘pregustatory’ and ‘postgustatory’ parameters. The courtship latency and the first chosen target depend on the chemoperception prior to the first gustatory contact, while the number of transitions, the CI, the DI, the frequency of males courting and the ‘global choice parameters’ allowed us to quantify the behaviour of the tester male after his first gustatory contact.

Except for the total CI, all parameters were related to the differential response of the tester male towards each of the two target flies. The total CI for both targets allowed us to check whether some combinations of targets induced a global change in the tester male’s ardour.

To date, the preference of a male tested in a choice procedure has always been scored using either DI or CI. The ‘global choice parameters’ allowed us to describe more completely the discriminatory ability of tester males. The comparison of these ‘global choice parameters’ with a same-sex pair of control targets revealed strong differences (Fig. 1g, h), whereas the DIs were not different (Fig. 1d). With control female pairs, although all testers courted, only 25% directed a similar courtship to both targets, whereas the other 75% continuously courted only one female. Conversely, with control male pairs, only 3% of males courted both male targets while 34% courted only one male. The weak DIs induced by a same-sex pair of control flies indicates that either: (1) tester males are randomly attracted by one target whose attractiveness is sufficient to prevent courtship of the other target, or that (2) both targets induce a weak response in tester males. Therefore, the ‘global choice parameters’ not only
reflect the robustness of the tester male choice but also provide information that completes the
‘number of transitions’ parameter to assess the courtship stability of the tester male.

Whereas in choice experiments, G66a-Gal4/UAS-tra males were unable to discriminate
between Tai and desat1 male targets (this study), in no-choice experiments they court Tai
more intensively than desat1 male targets (Lacaille et al. 2007, 2009). Several studies have
already shown that choice and no-choice mating experiments may yield diverging results (e.g.
Ryan & Rand 1993; Gupta & Sundaran 1994; Wade et al. 1995; Coyne et al. 2005). The
respective advantages and disadvantages of choice versus no-choice mating experiments are
still a matter of debate (see Casares et al. 2005; Noor & Ortiz-Barrientos 2006). While it has
been argued that the design of choice experiments is more realistic than that of no-choice
experiments (Spieth & Ringo 1983; Alipaz et al. 2005a, b), Coyne et al. (2005) suggested that
when Drosophila of different strains or species congregate, the flies could evaluate potential
mates individually and sequentially reject unfavourable partners. In D. bipectinata sexual
activity is different between choice and no-choice situations, with no relationship with the sex
to ratio (Singh & Sisodia 1999). Theoretically, the simultaneous presentation of two concurrent
stimuli allows us to obtain a more sensitive measure of the preference for one of them, while
their separate presentation provides a more rigorous test (Martin & Bateson 1993). However,
simultaneous presentation may be distracting to the subject, which may be ‘trapped’ by its
first choice whether or not it was random, and withdrawing from one target may be
incorrectly interpreted as approaching the other (Martin & Bateson 1993). However, mate
choice is a discrimination process between several objects. Consequently, only the
simultaneous presentation of two targets could yield a simplified but valuable representation
of what happens in the field where a subject could be trapped by, or diverted from, a stimulus
by another one. Both these effects could occur during male courtship with two target flies in
comparison with courtship towards a single female target and have to be taken into account in
evaluating male mate choice. Furthermore, to be more realistic future work should involve more than two target females belonging to various strains. The present work clearly shows that wild-type CS male flies are able to discriminate at a short distance between wild-type sex pheromones. We have also shown that they are able to discriminate between males and females of various strains, and that their preference could change after the first gustatory contact, especially towards female targets.

**<H2>Discrimination varies with tester male and target flies**

The three tester males showed very different abilities to discriminate male and female CS targets. While CS and 66a-Gal4/UAS-tra tester males preferred the CS target female, 66a-Gal4/+ males did not discriminate between the sexes. These differences were similar before and after the first gustatory contact.

With [T | CS] males, the three testers preferred the Tai male target, but with the [T | D] males 66a-Gal4/UAS-tra testers did not discriminate between the targets. In comparison with the other two male pairs, the [CS | D] pair induced the lowest CIs and discrimination. Moreover, the behavioural response induced by the [CS | D] pair varied with the male tester genotype: CS and 66a-Gal4/UAS-tra testers preferred the desat1 male target before and after gustatory contact while the 66a-Gal4/+ males preferred the CS target male before the first gustatory contact but showed no preference after this contact.

With female pairs, the response of tester males also varied before and after the first gustatory contact. With [CS | D] pairs, the three testers showed similar responses before and after gustatory contact: the CS female was always preferred. With [T | CS] pairs, males maintained a similar response before and after gustatory contact. However, the CS female target was preferred by CS and 66a-Gal4/+ testers whereas the Tai female target was preferred by 66a-Gal4/UAS-tra testers. Prior to the contact with [T | D] female pairs, 66a-Gal4/+ and CS
testers slightly preferred the Tai target whereas the 66a-Gal4/UAS-tra males slightly preferred the desat1 target. After gustatory contact, the respective preferences of the CS and the 66a-Gal4/UAS-tra males were enhanced whereas 66a-Gal4/+ testers did not show any preference.

**Role of cuticular hydrocarbons**

Except in the control experiment, the target flies diverged strongly in their main CHs (Table 1), some of which are known or suspected to play a pheromonal role in the behaviour of CS males (Table 1 and references therein).

The ability of the tester males to discriminate between CS male and female targets mostly relies on the sexual dimorphism of their principal CHs: 7-tricosene (7-T) in males and 7,11-dienes in females (Antony & Jallon 1982; Jallon 1984; Marcillac et al. 2005). Although it is not known whether *Drosophila* males can perceive female dienes, some information related to the perception of 7-T by *Drosophila* flies is available. In particular a subset of taste neurons expressing the Gr66a receptor is involved in the 7-T detection (Lacaille et al. 2007, 2009). This CH is suspected to play a reciprocal pheromonal role in both sexes: its gustatory perception tends to inhibit male courtship (Ferveur & Sureau 1996; Svetec & Ferveur 2005; Lacaille et al. 2007), whereas its olfactory perception by the female enhances her receptivity (Grillet et al. 2006). Although its low volatility may be increased by male motion, the amount of dispersed 7-T probably remains very low. This suggests that the olfactory perception of 7-T by females would entail a lower detection threshold than its gustatory perception by control males. This fits with two studies (Grillet et al. 2006; Lacaille et al. 2009). Our present results also indicate that 66a-Gal4/+ males weakly discriminate sex pheromones. This was reflected by the high number of transitions they showed between the two targets. Moreover the 66a-Gal4/+ males showed the same total CI as CS and 66a-Gal4/UAS-tra males, but their CI with the target male was twice that of the latter two males. Consequently, their CI with the female
target was lower than that shown by CS and 66a-Gal4/UAS-tra testers. Therefore, it is possible that the weak discrimination ability of 66a-Gal4/+ males is due to the decrease in their homosexual inhibition rather than in their heterosexual ardour.

The three tester males’ responses towards males varied after the first contact. The Tai target male was always preferred to the CS target male. While the preference of the CS and 66a-Gal4/UAS-tra tester males could be driven by the lower amount of 7-T produced by the Tai males than by the CS males (Table 1), the 66a-Gal4/+ testers could be stimulated by the higher amount of 7-pentacosene (7-P) produced by Tai males since they are unable to detect 7-T (Lacaille et al. 2007; Table 1). There have been suspicions that 7-P is an excitatory compound for CS males (Antony et al. 1985; Ferveur 1997). Furthermore, 7-P is thought to act synergistically with 9-pentacosene (9-P) to stimulate males attempting to copulate (Ferveur & Sureau 1996; Siwicki et al. 2005). This preference for Tai males over CS males also confirms a previous result obtained with a no-choice design: CS, Gr66a-Gal4/+ and Gr66a-Gal4/UAS-tra courted Tai target males more intensively than CS target males (Lacaille et al. 2007, 2009).

With [T | D] male pairs, the 66a-Gal4/UAS-tra tester males did not show any preferential response to either target, while CS and 66a-Gal4/+ tester males preferentially chose the Tai target. Since Tai and desat1 males produce very similar amounts of 7-T (Table 1), the preferential choice of CS and 66a-Gal4/+ testers could be induced by the highest amount of 7-P. On the other hand, 66a-Gal4/UAS-tra males, which seem to be very sensitive to 7-T, could be equally inhibited by the similar amounts of this substance carried by the two target males (Table 1).

Compared to other target combinations, [CS | D] male pairs induced the lowest CIs and DIs. This could be caused by (1) the presence of a large amount of 7-T on the CS target, and/or (2) the absence or low amounts of stimulating chemicals (7-P or 7,11-dienes) on both CS and
desat1 targets (Antony & Jallon 1982; Jallon 1984; Marcillac et al. 2005; Table 1). Before gustatory contact, CS and 66a-Gal4/UAS-tra testers preferred the desat1 male target, while the 66a-Gal4/+ testers preferred the CS male target. After the first gustatory contact, the 66a-Gal4/+ testers did not prefer either target, while the other two testers persisted in preferring the desat1 male target. Furthermore, the preference of the 66a-Gal4/UAS-tra testers for the desat1 male target was even enhanced after gustatory contact. These variations could also be related to the 7-T gustatory detection deficiency of the 66a-Gal4/+ tester males and to the gustatory oversensitivity of the 66a-Gal4/UAS-tra males to the same compound.

For male target pairs, the statistical analysis (stepwise DAs) of the cuticular profiles is consistent with our behavioural data: 7-T seems to be the most important CH used by tester males to discriminate either between CS males and females, or between CS and Tai or CS and desat1 males. However, 7-T seems not to be required by males to discriminate target females. Among pairs of target females, only the [CS | D] pairs induced clear behavioural responses in the three tester males: the CS target female was always preferred. CS females produce high levels of 7,11-dienes which are thought to stimulate male courtship (Antony & Jallon 1982; Jallon 1984; Ferveur & Sureau 1996), while the desat1 females mostly lack these CHs (Marcillac et al. 2005). The 7,11-dienes were clearly preferred by CS males in a choice experiment (Marcillac & Ferveur 2004). The preference of CS males for CS females also supports the general expectation of Dobzhansky & Mayr (Dobzhansky & Mayr 1944; Mayr & Dobzhansky 1945) that species and strains of Drosophila will generally show positive assortative mating.

With the [T | D] female pairs, except for a possible involvement of the 7,11 nonacosadiene (7,11-ND) in the preference of the CS tester for the Tai female target, our results could not be easily related to any main female CHs. The desat1 females possess larger amounts of 9-P and 7-P than the Tai females and these compounds are thought to stimulate male courtship
(Ferveur & Jallon 1996; Ferveur & Sureau 1996; Siwicki et al. 2005). Therefore, the preference of the CS males for the Tai females and of 66a-Gal4/UAS-tra males for the desat1 females could reflect a differential sensitivity to pentacosenes. The [T | CS] female pairs triggered responses that were maintained before and after the gustatory contact, but they differed between the three tester males: the CS female was strongly preferred by the 66a-Gal4/+ males and slightly preferred by the CS tester males, whereas the 66a-Gal4/UAS-tra tester males preferred the Tai female. Since CS females possess more dienes, 7-P and 7-T than Tai females, the excitatory effects of the two former substances could explain the preference of CS and 66a-Gal4/+ tester males. Conversely, the preference of the 66a-Gal4/UAS-tra males for Tai females could be related to their strong avoidance of 7-T in CS females. The preference pattern shown by the 66a-Gal4/UAS-tra males towards the three combinations of female pairs is more difficult to interpret: they preferred the CS females in [CS | D] pairs, the desat1 females in [T | D] pairs and the Tai females in [T | CS] pairs. This nonlinear pattern of preference suggests that discrimination involves more than one compound and that the feminization of the Gr66a neurons could lead to an alteration in the ‘sensorial representation’ of the pheromonal blend, especially resulting in the higher sensitivity to some components even at very low doses on the fly cuticle.

In conclusion, our results show that male mate choice exists in D. melanogaster and that male discrimination between potential mates could involve chemical cues perceived before and after the first gustatory contact. They also suggest that when a male is simultaneously presented with a choice of pheromonal cues produced by two potential sexual partners, he will use different cues depending on the CH patterns of both flies, and not the same cues for a given fly. Furthermore, his behavioural response could depend on additional effects caused by
the simultaneous perception of the two target flies (like the ‘trapping’ and ‘diverting’ effects explained above). This can lead to a nonlinear, thus unpredictable choice of a sexual partner. Moreover, the initial partner choice preference based on olfactory cues can, in some cases, be changed according to the choice of gustatory cues perceived later during the courtship ritual. Finally, the choice of a sexual partner based on pheromonal cues can clearly be modified in males with altered gustatory receptor neurons. This clearly indicates that the male fly’s peripheral gustatory system is essential for pheromonal detection and mate choice. Further experiments should help to elucidate the involvement of the olfactory system in male mate choice, for example using Or83b mutants (Larsson et al. 2001).

Acknowledgments

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References


FIGURE CAPTIONS

FIGURE 1: Behaviour that single CS tester males directed towards a pair of same-sex CS flies. A single CS tester male fly was given a choice between two decapitated target males or females from a wild-type strain (CS), under white light. Eight behavioural parameters reflecting the male’s ability to perceive both volatile and contact pheromones were measured, namely: (a) the courtship latency; (b) the first target chosen; (c) the courtship index towards each target; (d) the discrimination index; (e) the frequency of males courting each target; (f) the number of transitions between targets; (g) the global choice and (h) the global choice intensity. Except for percentages (b, e, g), parameters are expressed as their mean + SEM. Different lowercase letters indicate significant differences between target flies (Kruskal - Wallis analysis, completed by a Dunn's multiple pairwise comparison). ***P<0.001; z test in (e) and Mann - Whitney U test in (h). Different < and > symbols indicate significant differences between cells; chi-square test in (h). In (g), ‘ø’ indicates males that had no preference. See Methods for further details.

FIGURE 2: Ability of tester males of three genotypes (CS, 66a-Gal4/+ and 66a-Gal4/UAS-tra) to discriminate the sex pheromones of control flies. A single tester male fly was given a choice between a decapitated target male and a decapitated target female from a wild-type strain (CS), under red light. For parameters and statistics see Fig. 1, except for the global choice intensities (h) which were compared using the Kruskal - Wallis analysis (completed by a Dunn's multiple pairwise comparison). In (g), ‘ø’ indicates males that had no preference. See Methods for further details.
FIGURE 3: Ability of tester males of three genotypes (CS, 66a-Gal4/+ and 66a-Gal4/UAS-tra) to discriminate the quality of male cuticular pheromones. The three tester males were put with target males of three variant strains with different predominant cuticular hydrocarbons: CS, Tai (T), and desat1(D), under white light. Targets were paired in the three possible combinations: [CS | D], [T | D] and [T | CS]. For parameters and statistics see Figs 1 and 2. In (g), ‘ø’ indicates males that had no preference. See Methods for further details.

FIGURE 4: Ability of tester males of three genotypes (CS, 66a-Gal4/+ and 66a-Gal4/UAS-tra) to discriminate the quality of female cuticular pheromones. The three tester males were put, under white light, with target females of three variant strains that diverged in their predominant cuticular hydrocarbons: CS, Tai (T) and desat1(D). Targets were paired in the three possible combinations: [CS | D], [T | D] and [T | CS]. For parameters and statistics see Figs 1 and 2. In (g), ‘ø’ indicates males that had no preference. See Methods for further details.
### TABLE 1: Cuticular hydrocarbons (CHs) in the male and female flies from the Canton-S (CS), Tai and desat1 strains.

<table>
<thead>
<tr>
<th>CH</th>
<th>Males CS</th>
<th>Females CS</th>
<th>Sex pheromonal role in CS strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>desat1 Tai</td>
<td></td>
</tr>
<tr>
<td>7-T</td>
<td>707 ± 36</td>
<td>143 ± 9</td>
<td>166 ± 11 125 ± 15 tr 29 ± 2</td>
</tr>
<tr>
<td>5-T</td>
<td>46 ± 2</td>
<td>52 ± 3</td>
<td>25 ± 1   tr tr tr</td>
</tr>
<tr>
<td>9-P</td>
<td>101 ± 4</td>
<td>105 ± 3</td>
<td>238 ± 15 170 ± 7 52 ± 4 154 ± 8</td>
</tr>
<tr>
<td>7-P</td>
<td>125 ± 12</td>
<td>1107 ± 62</td>
<td>56 ± 3   158 ± 15 11 ± 1 66 ± 5</td>
</tr>
<tr>
<td>7,11-HD</td>
<td>tr</td>
<td>tr</td>
<td>tr 469 ± 35 127 ± 8 109 ± 13</td>
</tr>
<tr>
<td>7,11-ND</td>
<td>tr</td>
<td>tr</td>
<td>tr 190 ± 22 129 ± 12 72 ± 122</td>
</tr>
</tbody>
</table>

Inhibits male courtship
Enhances female receptivity
Inhibits male courtship
Acts in synergy with 7-P to stimulate attempting to copulate
Acts in synergy with 7,11-dienes to stimulate male courtship
Acts in synergy with 9-P to stimulate attempting to copulate
Stimulates male courtship
Stimulates male courtship

TABLE 2: Discriminant analyses (DAs) using either the sex or the strain as a qualitative variable and the absolute amounts of uncorrelated CHs as quantitative variables (without selection and with a forward stepwise selection of the quantitative variables)

<table>
<thead>
<tr>
<th>Pairs</th>
<th>DA % Correctly classified</th>
<th>Stepwise forward DA Selected CHs (100% well-classified individuals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterosexual pair</td>
<td>[100.0</td>
<td>100.0]</td>
</tr>
<tr>
<td>Male pairs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[CS</td>
<td>D]</td>
<td>[73.3</td>
</tr>
<tr>
<td>[T</td>
<td>D]</td>
<td>[46.7</td>
</tr>
<tr>
<td>[T</td>
<td>CS]</td>
<td>[66.7</td>
</tr>
<tr>
<td>Female pairs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[CS</td>
<td>D]</td>
<td>[73.3</td>
</tr>
<tr>
<td>[T</td>
<td>D]</td>
<td>[40.0</td>
</tr>
<tr>
<td>[T</td>
<td>CS]</td>
<td>[66.7</td>
</tr>
</tbody>
</table>

The results of the different DAs are summarized by their confusion matrices after cross-validation, and by the selected CHs for forward stepwise DA. The statistical method is detailed in the Methods. Selected CHs are listed according to their elution order: 6-D = 6-docosene; 9-T = 9-tricosene; 7-T = 7-tricosene; 23-L = n-tricosane; 8-Te = 8-tetracosene; 25-Br = methyl-branched pentacosane; 9-P = 9-pentacosene; 5-P = 5-pentacosene; 26-L = n-hexacosane; Br-H = branched-heptacosene; 27-Br = methyl-branched heptacosane; 7-H = 7-heptacosene; 27-L = n-heptacosane; 7,11-ND = 7,11-nonacosenediene; 7-N = 7-nonacosene.
Target male genotypes: CS Tai desat1

Tester male genotypes: CS 66a/tra

Courtship latency (s)

First choice (%)

Discrimination index

Global choice intensity

Percentage of courting males (%)

Number of transitions

Global choice (%)

Chosen target: [CS | D] [T | D] [T | CS]

Target males: CS 66a/CS 66a/tra

Target male genotypes: CS desat1 Tai Tester male genotypes: CS 66a/CS 66a/tra
### Data Table

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>66a/CS</th>
<th>66a/tra</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Courtship Latency (s)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Discrimination Index</strong></td>
<td>-100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><strong>Courtship Index</strong></td>
<td>0</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td><strong>First Choice (%)</strong></td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><strong>Global Choice (%)</strong></td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><strong>Percentage of Courting Females (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of Transitions</strong></td>
<td>0.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Global Choice Intensity</strong></td>
<td>0</td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

### Notes
- **Target Female Genotypes**: CS Tai, desat1
- **Tester Male Genotypes**: CS 66a/tra
- **Target Females**: CS 66a/CS 66a/tra

### Figures
- **(a)**: Bar graphs showing courtship latency for different tester genotypes.
- **(b)**: Bar graphs showing first choice for different target genotypes.
- **(c)**: Bar graphs showing courtship index for different tester genotypes.
- **(d)**: Bar graphs showing discrimination index for different target genotypes.
- **(e)**: Bar graphs showing percentage of courting females for different tester genotypes.
- **(f)**: Bar graphs showing number of transitions for different target genotypes.