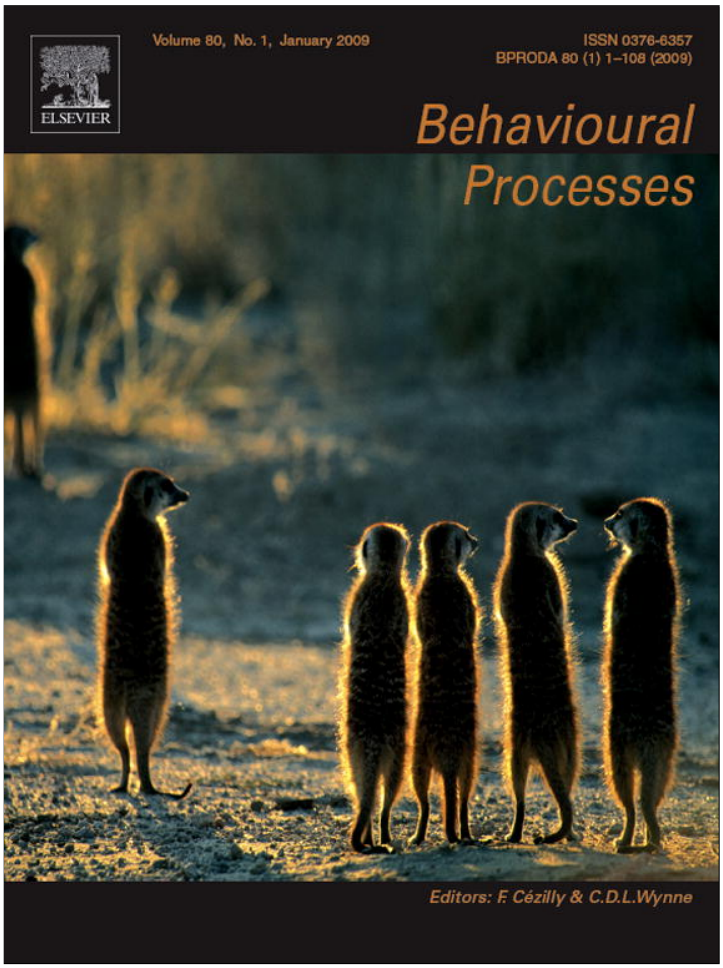

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Signalling components of the house mouse mate recognition system

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ABSTRACT

Subspecies-specific mate recognition may represent significant barrier to gene flow between diverged genomes potentially leading to speciation. In the house mouse, assortative mating involves the coevolution of several signals and receptors. We compared signalling ability of bedding material, faeces, urine, saliva, salivary androgen binding proteins (ABP) and combinations of urine with saliva and urine with ABP in mate choice in two wild-derived inbred strains (one of *Mus musculus musculus* and one of *Mus musculus domesticus* origin). We observed high levels of variation in assortative preferences between the two strains and sexes. The strongest preferences were observed in *M. m. musculus*-derived individuals in tests where urine was present either alone or as part of a composite signal target. *M. m. domesticus*-derived mice displayed strain-specific preferences for faeces. Saliva was the least preferred stimulus in both strains and sexes. No effect of two-compound cues was detected. We conclude that there is divergence across both the stimulus and preference parts of the recognition system for both house mouse strains. Of the tested stimuli, those that have the capacity to carry a signal for extended periods under natural conditions (such as urine and faeces) seem to be the most important substances in strain-specific recognition.

1. Introduction

The traditional view of mating signal evolution suggests that certain features of the mate choice signals can be used for species recognition. While stabilizing selection decreases variance in these signals making them reliable species-specific indicators, disruptive selection increases divergence of these signals between isolated groups (Butlin, 1995; Bridle and Ritchie, 2001). In theory, only individuals of the same species are able to perform the signal-response sequence necessary to achieve mating whereas this sequence will not be completed successfully in interspecific pairs (Butlin and Ritchie, 1994). Thus the species-specific mate recognition signals can serve as a significant barrier between diverged genomes, prevent their mixing, and eventually leading to complete speciation. Divergent signals involved in behavioural and reproductive isolation, including visual, olfactory, chemical or tactile cues, have been described in many closely related species (reviewed in Ptacek, 2000; Coyne and Orr, 2004).

One possible approach to search for species-specific signals is to study sister taxa where complete isolation has not yet been achieved. In these cases, selection can promote the divergence between recognition systems in areas of their secondary contact through the process of reinforcement (Butlin, 1995). A suitable model for such a kind of study is the house mouse, whose two subspecies, *Mus musculus musculus* and *M. m. domesticus*, mate and hybridise, forming a long and narrow hybrid zone in Europe (Boursot et al., 1993; Macholán et al., 2003; Raufaste et al., 2005; Macholán et al., 2007). In this zone, behavioural isolation, acting through assortative mating, could play an important role as a prezygotic barrier reinforcing selection against hybrids, eventually leading to speciation between the subspecies (Dod et al., 1993, 2005; Karn et al., 2002; Smadja et al., 2004; Ganem et al., 2008).

The main components of the signal–receptor system assumed to be involved in assortative mate choice in the house mouse include chemical signalling using excretory products such as urine, faeces and glandular exudates. This system is paramount for social relationships, survival and reproduction (reviewed by Beauchamp and Yamazaki, 2003 and Brennan and Kendrick, 2006). Olfactory cues not only convey information including sex, reproductive and health status (Ehman and Scott, 2001; Kavaliers et al., 2003, 2004, 2005), competitive ability, and territory ownership (Rich and Hurst, 1999; Beynon and Hurst, 2003), but also individual identity, such as genotype (Penn and Potts, 1999; Heth et al., 2003; Thom and Hurst, 2004; Thom et al., 2008), familiarity or kinship (Hurst et al., 2001, 2005; Todrank et al., 2005; Sherborne et al., 2007). In addition to the essential role of urine in mouse communication (Novotny, 2003), the impact of body pheromones (Röck et al., 2006) and exocrine gland secretions, applied mainly on the facial area (such as saliva

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or tears) or around the anus, has recently been highlighted (Luo et al., 2003; Kimoto et al., 2005). Thus, the use of different odour cues may present a complex signalling system whose components may play specific roles either in time (e.g. as long-lasting scent marks or as recognition signals on close contact between individuals) or in transmitted information (e.g. during individual or (sub)species-specific recognition).

In the context of the *musculus-domesticus* recognition, the ability to discriminate and choose consubspecifics has been repeatedly demonstrated using bedding (Munclinger and Frynta, 2000; Christophe and Baudoin, 1998; Smadja and Ganem, 2002), urine (Smadja and Ganem, 2002, 2005; Smadja et al., 2004) and salivary ABP (Laukaitis et al., 1997; Talley et al., 2001) as signal stimuli. However, these studies have mainly been focused on discrimination and preference *per se*, not directly on the role of different stimuli involved in complex subspecies-specific odour type. For example, Smadja and Ganem (2002) demonstrated that subspecies-specific recognition occurs through urinary signals and these have been diverged and reinforced in the secondary hybrid zone between the two subspecies (Smadja and Ganem, 2005, 2008). Similarly, Laukaitis et al. (1997) and Talley et al. (2001) suggested one of the androgen binding protein (ABP) subunits, *Abpa*, as a subspecies-specific signal leading to prezygotic isolation between the two mouse taxa. However these data were not fully confirmed in natural populations (Dod et al., 2005; Bímová et al., 2005; Macholán et al., in press) indicating this element to be only one element of a more complex signalling system. Such a system may involve different stimuli that may be combined to modulate, complete or reinforce the transmitted information (Wyatt, 2003). Understanding an entire system (i.e. the response of a receiver to a complex signal) leading to assortative mating requires an approach focused on the analysis of a number of more, but basic components of this system and their possible combined effect.

In this study, we compared the role of five different odour stimuli potentially involved in subspecific signalling in *M. m. musculus* and *M. m. domesticus*. Since higher variation in behavioural responses has been reported in wild animals compared to inbred ones (Bímová et al., 2005), as a first approximation to the analyses of the complex signalling system in the house mouse hybrid zone we used two inbred strains, each derived from wild *M. m. musculus* and *M. m. domesticus* population, respectively (Piálek et al., 2008). The use of repeated two-way choice tests of the same individuals allowed us to directly compare the signalling power of presented targets. This is the first analysis comparing different signal components and their possible combined effect.

2. Materials and methods

2.1. Animals

Subject mice originated from two inbred strains (22 females and 22 males from each strain): STRA, derived from a wild population of *M. m. domesticus* from Straas [50°11′N, 11°46′E], Germany, and BULS, derived from a *M. m. musculus* population from Buškovice [50°13′N, 13°21′E], Czech Republic (see Piálek et al., 2008, for details of strain characteristics). The strains were maintained in the breeding facility of the Institute of Vertebrate Biology, ASCR, and were propagated from wild ancestral pairs by strict brother–sister mating for 14 generations.

In each simple two-way choice test the subject individual was presented with the stimuli from two pools: one '*musculus*' and one '*domesticus*' from animals of the opposite sex. As the subject animals were inbred there was a risk of inbreeding avoidance leading to preference for unfamiliar strain (Penn, 2002; Sherborne et al., 2007). Therefore we used two other inbred strains derived from the same wild populations as sources of stimuli (except for sources of ABP; see below): BUSNA, representing *M. m. musculus*, and STLT, representing *M. m. domesticus*, respectively (for more details see Piálek et al., 2008), at F14 generation of brother–sister mating. In order to eliminate the effect of individuality in the stimuli, i.e. to ensure the observed preferences to be genuinely strain-specific, we pooled the stimuli from at least five stimulus individuals of the same sex and strain.

All mice were housed in Perspex cages (16 cm × 28 cm × 15 cm) with sawdust bedding under the following constant conditions: 14:10 L:D (i.e. light on between 06.30 and 20.30), 22 °C, approximately 40% humidity; standard mouse pelleted food (ST1, VELAZ, Prague, Czech Republic) and tap water were available *ad libitum*. After weaning at the age of 20 days, mice were placed in cages either separately or in pairs with a littermate of the same sex and tested as adults older then 60 days. In the case of males housed with a littermate, males were isolated 5 days before the first test and were housed singly until the end of the study to prevent a potential effect of social dominance between males.

All mice used in our study descended from inbred strains and were derived specifically for purposes of this study. At the end of the study, the mice were sacrificed by cervical dislocation. The whole study followed the experimental protocol (No. 5/05) approved by Institutional Committee and Czech Academy of Sciences Committee for animal welfare. The breeding facility has been licensed (3245/2003-1020) for keeping small mammals according to Czech law since 2000 and the first author holds a license (V/1/2005/03) for experimental work on vertebrates in accordance with Czech law.

2.2. Signal stimuli

To asses the signalling power of different components of the overall mouse odour we tested sexual preferences in a set of repeated two-way choice tests on the same individual for the following stimuli: soiled bedding, faeces, urine, saliva, *Abpa*-specific saliva (hereafter referred to as ABP) and combinations of urine with saliva (urine + saliva) and urine with ABP (urine + ABP), respectively. The two combinations were chosen based on previous evidences of the role of urine and ABP as signals in house mouse subspecies-specific discrimination (Laukaitis et al., 1997; Smadja et al., 2004; Ganem et al., 2008). Tested stimuli represented both long-lasting signals (such as those contained in bedding, urine and faeces) and signals potentially used when two interacting individuals are in close contact (stimuli present in saliva and ABP) and varied from sources potentially presenting a variety of stimuli (bedding), to sources containing specific stimuli (e.g. urine or saliva involving mainly volatile pheromones, MUPs and MHC) finally to ABP representing a single genotype difference.

2.2.1. Preparation of soiled bedding pools

Five males and five females from each 'signal' strain (BUSNA and STLT) were placed singly in cages containing 50 g of sterile bedding. After 10 days the bedding was pooled for the same strain and sex and divided into 15 g portions in sterile plastic bags and frozen at −80 °C. One portion of bedding per signal strain was defrosted fifteen minutes prior to each experiment and put into the peripheral boxes attached to the testing apparatus (see below).

2.2.2. Preparation of faeces pools

Faeces were collected in clean sterile cages, pooled from at least five individuals of the same signal strain and sex and stored in sterile tubes at −80 °C. We used only faeces that had not come into contact with urine or other signal stimuli. For each experiment, 200 mg of pooled faeces from each signal strain were used

as stimuli. Since the animals were kept in standard conditions the potential effects of parasite infection, disease and nourishment on the resulting signal differences were minimized. Fifteen minutes prior to each test the faeces were defrosted and placed on filter paper strip (2.5 cm × 20 cm).

2.2.3. Preparation of urine pools

Urine was collected in clean sterile cages and pooled from at least five animals of the same signal strain and sex and stored at −80 °C. Prior to each experiment, 10 μl of urine form each signal strain was defrosted and spotted in the middle of a sterile filter paper strip, dried for 15 min at room temperature and positioned in the Y-maze.

2.2.4. Subspecific saliva and ABP

Saliva from both subspecies of the house mouse and ABP were collected by the isoproterenol-stimulated salivation method described by Karn (1981). The former stimulus was represented by pooled saliva from at least five individuals of the same sex and signal strain, while the ABP was collected from at least five individuals of strains with the same genetic background, but differing only in their *Abpa* allele: the $Abpa^a$ allele is carried by the C3H/HeJ strain (purchased from ANLAB, Prague), whereas the $Abpa^b$ allele is carried by the $Abpa^b$-congenic strain established from DBA mice backcrossed to C3H/HeJ (for further details see Laukaitis et al., 1997) provided by R.C. Karn (Butler University, Indianapolis, Indiana). By using these two types of saliva stimuli, we could study and compare the overall role of mouse saliva, and more specifically, the signalling potential of the *Abpa* gene. All saliva was stored at −80 °C. Prior to testing, 10 μl of defrosted saliva were spotted in the middle of a sterile strip of the filter paper and left to dry at room temperature for 15 min before being positioned in the Y-maze.

2.2.5. Combinations of stimuli

Three different homosubspecific combinations of urine and saliva were used: (1) 5 μl of urine combined with 5 μl of saliva from the same signal strain STLT or BUSNA ('urine + saliva'); (2) 5 μl of urine and 5 μl of saliva from an *Abpa*-specific strain ('urine + ABP'); (3) for females only, 10 μl of urine and 10 μl of saliva from the same signal strain. This final combination was used to validate that our results were not influenced by an insufficient amount of the tested stimuli. Urine and saliva were spotted together on the same strip of the filter paper.

2.3. Experimental procedure

Accordingly to similar studies, frequently using olfactory tests as a representative of mate choice preference (Smadja and Ganem, 2002; Talley et al., 2001; Brennan and Kendrick, 2006) we used a simple two-way choice tests in a Y-maze. The testing apparatus consisted of a central box (35 cm × 25 cm × 13 cm) connected to a Y-maze (diameter: 5 cm; stem length: 35 cm; side arms length: 23 cm) (see Bímová et al., 2005 for the apparatus design). In order to test for preferences for soiled bedding, two peripheral boxes were connected to the ends of the side arms (35 cm × 25 cm × 13 cm) and direct contact with this stimulus was achieved by allowing the tested mouse to enter and investigate the bedding placed in the peripheral test boxes. All other tested stimuli were placed in the middle of a sterile strip of filter paper (1.5 cm × 20 cm) positioned in the bottom of each side arm. One-way air circulation in the Y-maze from the ends of the side arms to the central box ensured that air-borne chemical signals were continuously present at the branching point of the side arms during the whole experiment. The air circulation was forced by an electric valve placed in a neighbouring room to minimize acoustic disturbance. In order to control for laterality, the position of odour sources in the arms of the Y-maze was changed randomly in successive tests. Between each test we cleaned the Y-maze with 70% ethanol and boxes with $NaClO_4$ solution (<5%), thoroughly rinsed them with tap water. All tests were performed during the light phase of the day. As the experimental protocol involved repeated preference tests of the same individual for different stimuli, the experiments involving the same tested animal were done at 14-day intervals.

At the beginning of each test the tested animal was weighed and placed in the central box where it was allowed to habituate for at least 15 min. After habituation, the stimuli were positioned and the door leading from the central box to Y-maze was opened allowing the animal to enter the maze. We recorded the individual's behaviour for 5 min starting immediately after it left the central box for the first time. During the test the mouse was left free to investigate the apparatus and stimuli. Contact with the stimulus was recorded when the mouse sniffed, licked or chewed the stimulus. The behaviour was recorded and analysed using Observer software (Noldus et al., 2000). All female mice were scored for their sexual receptivity (vaginal smears were performed immediately at the end of each series of test) and subsequently divided into two groups: receptive females (in proestrus or oestrus phase of the cycle) and non-receptive females (in all other cycle phases). However no effect of oestrus period on sexual preferences was found in all tested signals (ANOVA; beddings: $F_{(1,40)} = 2.187$, $P = 0.147$; faeces: $F_{(1,40)} = 3.71$, $P = 0.061$; urine: $F_{(1,37)} = 0.018$, $P = 0.893$; saliva: $F_{(1,40)} = 0.910$, $P = 0.346$; ABP: $F_{(1,18)} = 1.271$, $P = 0.274$; urine + saliva: $F_{(1,25)} = 0.007$, $P = 0.936$; urine + ABP: $F_{(1,39)} = 0.603$, $P = 0.442$) and thus non-receptive females were not excluded from further analyses.

2.4. Statistical analysis of signal preferences

Since mice displayed exploratory behaviour similar to that previously described (Smadja and Ganem, 2002; Bímová et al., 2005), we used the same calculation as these authors to estimate the preference coefficient as the difference in time spent in contact with either of the stimuli as $R_{signal} = T_{ho}/(T_{ho} + T_{het})$, where T_{ho} is the time spent sniffing the signal of the same strain and T_{het} is the time spent sniffing the signal of the opposite strain. The coefficient ranges from 0 to 1 where $R_{signal} < 0.5$ indicates preference for the strain derived from opposite subspecies (hereafter referred as disassortative preference), and $R_{signal} > 0.5$ preference for the signal strain derived from the same subspecies (hereafter assortative preference); $R_{signal} = 0.5$ corresponds to the absence of preference. More time may be necessary to process information about individual cues from complex stimuli, thus more interest may be paid to more informative stimuli. To distinguish the interest in more complex information present in the stimuli from the preference we measured, as an additional variable, the total time spent by sniffing either stimulus ($T_{signal} = T_{ho} + T_{het}$).

The normality of the distributions of the variable R_{signal} and T_{signal} was checked using the Kolmogorov–Smirnov test, we found no significant deviation of R_{signal} and T_{signal} from normality. The overall model tested the significance of the effect of strain:sex:signal interaction on R_{signal} using linear mixed-effect models as implemented in the R software (Crawley, 2007). Because each individual was tested several times, random structure with the effect of individual (85 in total) nested within sex and strain was assumed in the model (Crawley, 2007). The model involving the three-way interaction was compared with model involving main effects and all two-way interactions using maximum likelihood approach. In the separate analyses conducted for each strain–sex combination, individual was involved as a random effect to avoid pseudoreplication (Crawley, 2007). The significance of the categor-

ical explanatory variable signal type was based on the change in deviance between the full and reduced models, distributed as $\chi 2$ with degrees of freedom equal to the difference in the degrees of freedom between the null model and model with the term in question incorporated. *A posteriori* simplification of factor levels (different signal type) was applied when the model suggested a significant effect of signal type on R_{signal} (also Crawley, 2007). Each model used only individuals that have displayed preference (e.g. entered the Y-maze and sniffed both signal pools in each repeated test) for all compared signals. Thus successive analyses differ in number of tested individuals.

Previous analysis tested the role of signal type on mice preference within sex and strain. We used Student's *t*-test to test for statistically significant preferences (the difference of R_{signal} from 0.5) separately for each signal type, sex and strain. Tests were two-tailed with type I error set to $\alpha = 0.05$; the level of significance was adjusted with a Bonferroni correction as the procedure involved multiple testing of the same hypothesis on repeated samples ($\alpha = 0.05/28$ repetitive tests = 0.0018). Analyses were performed using the R 6.0.2 (Crawley, 2007) and Statistica 6.0 software package (SAS Institute, 2002).

3. Results

3.1. Signalling power of individual stimuli

The model involving the three-way interaction of strain:sex:signal explained the variability in signal preferences much better than model without this interaction ($\chi^2 = 15.31$, d.f. = 4, $P = 0.004$), indicating varying strength and direction of signal preferences among strains and sexes. Consequently, the effect of signal types on preferences was evaluated separately for males and females and for each strain (Table 1). The analysis revealed a significant difference among preferences for particular signals in all four groups (Fig. 1). *A posteriori* simplification allowed to pool together signals bedding, faeces and urine in BULS males (effect of signal on preferences: $\chi^2 = 17.77$, d.f. = 4, $P = 0.001$; change to deviance after pooling together these signals: $\chi^2 = 2.90$, d.f. = 2, $P = 0.234$), bedding with faeces and saliva with ABP in BULS females (effect of signal: $\chi^2 = 16.27$, d.f. = 4, $P = 0.003$, change to deviance after pooling together these signals: $\chi^2 = 4.32$, d.f. = 3, $P = 0.229$), all factor levels except of bedding in STRA males (effect of signal: $\chi^2 = 20.93$, d.f. = 4, $P < 0.001$, change to deviance after pooling together all signals except of beddings: $\chi^2 = 1.38$, d.f. = 3, $P = 0.711$), and bedding with urine and ABP in STRA females (effect of signal: $\chi^2 = 18.08$, d.f. = 4, $P < 0.001$, change to deviance after pooling together these signals: $\chi^2 = 0.07$, d.f. = 2, $P = 0.964$). Other simplifications to the models were not supported (all $P < 0.05$) (Fig. 1).

Student's *t* tests of preferences for individual signals (Table 1) analysed separately per sex and strain revealed the strongest significant assortative preferences (after Bonferroni correction) in BULS strain in tests where the stimulus was urine (males: $\mu = 0.730$; $t = 6.61$; d.f. = 20, $p < 0.0001$ and females ($\mu = 0.725$; $t = 5.94$; d.f. = 21, $p < 0.0001$) and than in males for bedding as signal ($\mu = 0.709$; $t = 4.10$; d.f. = 20, $p = 0.0006$). Assortative significant preferences in STRA strain were found only in females in tests with faeces as signal ($\mu = 0.713$; $t = 6.42$; d.f. = 19, $p < 0.0001$). Contrary STRA males displayed disassortative preferences for bedding ($\mu = 0.284$; $t = -4.04$; d.f. = 21, $p = 0.0006$ (Fig. 1). In all other tests the significant level adjusted by Bonferroni correction to 0.0018 was not reached (Table 1). The analyses of the total time spent by signal sniffing (T_{signal}) (Table 1) revealed the lowest interest paid to salivary signals especially in the case of ABP in BULS mice. Females of both strains spent the longest time sniffing bedding and paid minimum attention to ABP, while males of both strains spent a similar amount of time sniffing bedding, faeces and urinary signals and less time sniffing the saliva stimuli (saliva and ABP).

3.2. The effect of combined stimuli

3.2.1. The effect of stimulus volume

No significant differences in female behaviour was detected for the two volumes (10 μl and 20 μl) of combined stimuli (urine + saliva) for either strain (*t*-test for dependent samples, STRA females: $t_{(N=16)} = 0.04$, $p = 0.971$; BULS females: $t_{(N=10)} = 1.17$, $p = 0.272$). Similar results were obtained for the total time spent sniffing stimuli in BULS females (*t*-test for dependent samples, $t_{(N=10)} = -0.37$, $p = 0.723$); however, STRA females spent less time sniffing the double volume of the stimulus (*t*-test for dependent samples, $t_{(N=16)} = 2.46$, $p = 0.027$). The level of significance for *t*-tests for dependent samples was adjusted by Bonferroni correction to 0.0125 ($\alpha = 0.05/4$ repetitive tests). Based on these results, the stimulus was always presented in a 10 μl volume in all other tests (for both single and combined stimuli).

3.2.2. Combination of urine with saliva

Models for each sex–strain combination included signal with three levels (urine, saliva, urine + saliva) as explanatory variable for R_{signal}. A combination of urine + saliva did not differ in preferences from pure urine by BULS males (change to deviance after pooling together these two factor levels: $\chi^2 = 0.56$, d.f. = 1, $P = 0.455$), but both differed from pure saliva ($\chi^2 = 16.67$, d.f. = 1, $P < 0.001$). The same pattern was apparent for BULS females (change to deviance after combining urine and urine + saliva: $\chi^2 = 0.02$, d.f. = 1, $P = 0.899$; the effect of urine and urine + saliva combined vs. pure saliva: $\chi^2 = 12.06$, d.f. = 1, $P = 0.001$). STRA males did not differ in their preferences for saliva, urine and a combined stimulus urine + saliva ($\chi^2 = 2.65$, d.f. = 2, $P = 0.266$) and the same was true for females ($\chi^2 = 5.30$, d.f. = 1, $P = 0.071$).

Student's *t*-tests of preferences for combined signal of urine and saliva (Table 1) revealed significant (after Bonferroni correction) assortative preferences only in BULS males ($\mu = 0.777$; $t = 9.24$; d.f. = 20, $p < 0.0001$) (Fig. 1). Females spend similar time by sniffing individual signals (urine and saliva) and the combined signal (urine + saliva), but males of both strains sniff urine longer than saliva or urine + saliva (Table 1). Thus, the combination of the two stimuli (urine + saliva) appears to have no significant effect either on preference or the overall interest in a stimulus.

3.2.3. Combination of urine with ABP

Models for each sex–strain combination included signal with three levels (urine, ABP, urine + ABP) as explanatory variable for R_{signal}. A combination of urine + ABP did not differ in preferences from pure urine by BULS males (change to deviance after pooling together these two factor levels: $\chi^2 = 0.12$, d.f. = 1, $P = 0.734$), but both differed from pure ABP ($\chi^2 = 16.77$, d.f. = 1, $P < 0.001$). The same pattern in R_{signal} preferences was found in BULS females (change to deviance after combining urine and urine + saliva: $\chi^2 = 0.36$, d.f. = 1, $P = 0.548$; the effect of urine and urine + ABP combined vs. pure ABP: $\chi^2 = 23.82$, d.f. = 1, $P < 0.001$). STRA males did not differ in their preferences for ABP, urine and a combined stimulus urine + ABP ($\chi^2 = 3.10$, d.f. = 2, $P = 0.213$). However, for females the urine and ABP could be combined ($\chi^2 = 0.33$, d.f. = 1, $P = 0.568$) and the reaction to urine and ABP presented separately differed from compositional ABP + urine signal ($\chi^2 = 7.48$, d.f. = 1, $P = 0.006$).

Student's *t*-tests of preferences for combination of urine and ABP (Table 1) revealed significant (after Bonferroni correction) assortative preferences only in BULS males ($\mu = 0.753$; $t = 4.998$; d.f. = 18,

Table 1
Total time spend by sniffing signals (T_{signal}: mean, S.E.) and the coefficients of preference (R_{signal}: mean, S.E.) assessed for individual signals and combinations separately per sex and strain

Strain	Sex	Signal	T_{signal}: mean (S.E.)	R_{signal}: mean (S.E.)	N	t-Value	d.f.	P-value
BULS	F	Bedding	32.409 (4.641)	0.597 (0.057)	22	1.725	21	0.0993
BULS	F	Faeces	14.182 (1.384)	0.545 (0.036)	22	1.243	21	0.2274
BULS	F	Urine	10.682 (0.963)	**0.725 (0.038)**	**22**	**5.944**	**21**	**0.0000**
BULS	F	Saliva	5.909 (0.873)	0.490 (0.059)	22	−0.169	21	0.8671
BULS	F	ABP	1.091 (0.415)	0.439 (0.082)	11	−0.740	10	0.4762
BULS	F	Urine + saliva	6.909 (1.587)	0.714 (0.074)	11	2.899	10	0.0159
BULS	F	Urine + ABP	6.524 (1.457)	0.641 (0.066)	21	2.131	20	0.0457
BULS	M	Bedding	23.381 (3.569)	**0.709 (0.051)**	**21**	**4.101**	**20**	**0.0006**
BULS	M	Faeces	22.619 (2.906)	0.602 (0.042)	21	2.421	20	0.0251
BULS	M	Urine	21.333 (2.929)	**0.730 (0.035)**	**21**	**6.609**	**20**	**0.0000**
BULS	M	Saliva	6.143 (1.998)	0.507 (0.070)	21	0.095	20	0.9253
BULS	M	ABP	1.789 (0.469)	0.436 (0.085)	19	−0.752	18	0.4616
BULS	M	Urine + saliva	16.905 (2.091)	**0.777 (0.030)**	**21**	**9.243**	**20**	**0.0000**
BULS	M	Urine + ABP	14.316 (1.969)	**0.753 (0.051)**	**19**	**4.998**	**18**	**0.0001**
STRA	F	Bedding	39.550 (3.929)	0.552 (0.064)	20	0.808	19	0.4292
STRA	F	Faeces	11.350 (1.152)	**0.713 (0.033)**	**20**	**6.417**	**19**	**0.0000**
STRA	F	Urine	8.200 (0.583)	0.564 (0.047)	20	1.364	19	0.1885
STRA	F	Saliva	6.700 (1.150)	0.370 (0.075)	20	−1.735	19	0.0989
STRA	F	ABP	6.556 (1.556)	0.522 (0.112)	9	0.197	8	0.8491
STRA	F	Urine + saliva	8.500 (1.360)	0.441 (0.061)	16	−0.970	15	0.3476
STRA	F	Urine + ABP	5.550 (0.939)	0.681 (0.063)	20	2.882	19	0.0095
STRA	M	Bedding	14.455 (2.492)	**0.284 (0.054)**	**22**	**−4.035**	**21**	**0.0006**
STRA	M	Faeces	21.000 (1.902)	0.555 (0.038)	22	1.435	21	0.1659
STRA	M	Urine	26.182 (1.255)	0.518 (0.026)	22	0.682	21	0.5027
STRA	M	Saliva	5.667 (0.871)	0.535 (0.058)	21	0.599	20	0.5561
STRA	M	ABP	9.864 (1.957)	0.483 (0.065)	22	−0.265	21	0.7939
STRA	M	Urine + saliva	13.000 (1.211)	0.448 (0.033)	22	−1.582	21	0.1286
STRA	M	Urine + ABP	10.909 (0.853)	**0.350 (0.036)**	**22**	**−4.220**	**21**	**0.0004**

Number of individuals (N), t-statistics (Student's t-test for R_{signal}, $H_0: \mu_0 = 0.5$) and corresponding P values are given for each test. The level of significance was adjusted using a Bonferroni correction to 0.0018. Statistically significant preferences are indicated in bold. Positive t values indicate assortative preferences and negative t values indicate disassortative preferences.

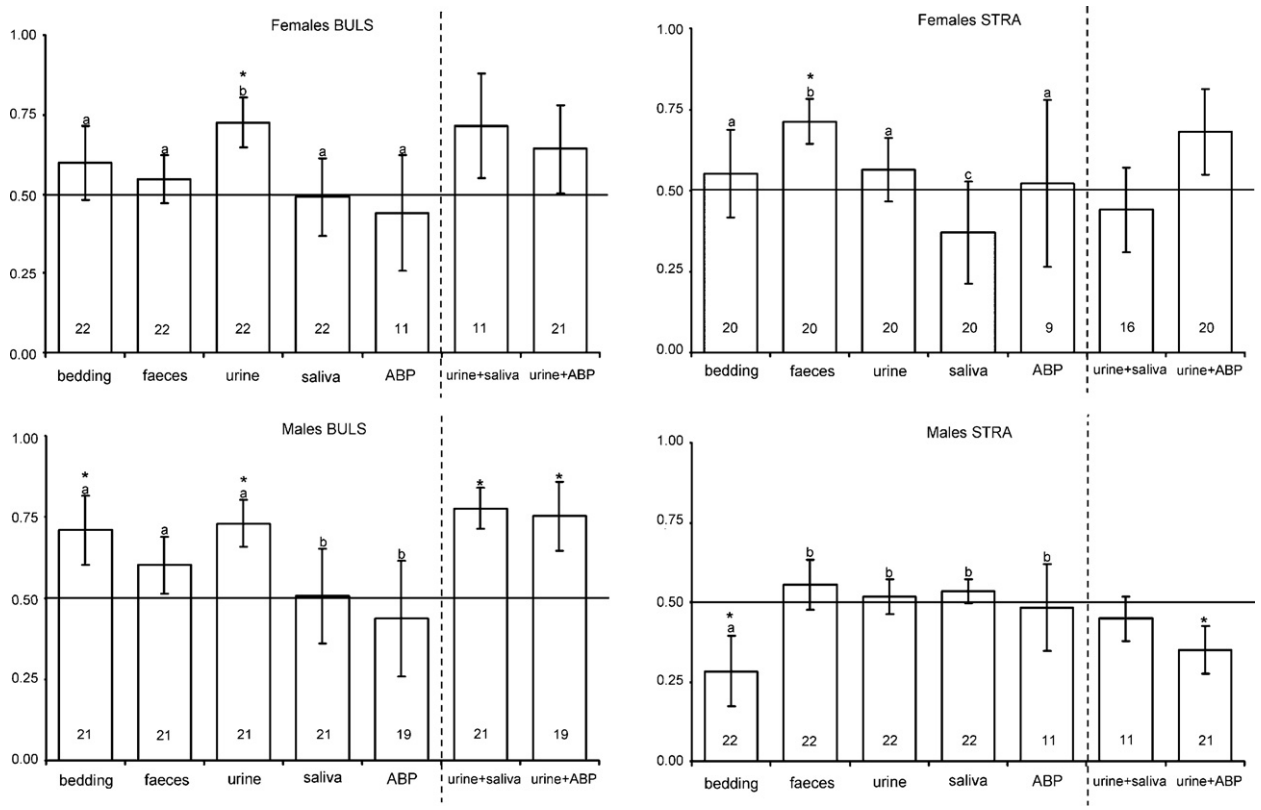

Fig. 1. Mean coefficients of preference of different individual stimuli and combined stimuli (in each panel separated by dashed vertical line) expressed separately for each strain and sex. Vertical bars represent 95% confidence interval of mean R_{signal}. Values above the line at $R_{signal} = 0.5$ (indicated in bold) represent assortative preference; values below this line represent disassortative preference. Statistically significant preferences (t-test; $H_0: \mu = 0.5$; Bonferroni adjustment of level of significance to 0.0018) are indicated with asterisks where $^*P < 0.0001$. Homogeneous groups of preferred signals tested by whole model are indicated with the same letter. Numbers of tested individuals in each group are indicated inside the columns.

$p = 0.0001$) (Fig. 1). Contrary STRA males displayed significant disassortative preferences ($\mu = 0.350$; $t = -4.22$, d.f. = 21, $p = 0.0004$). The total time spent by sniffing the combined signal (urine + ABP) was not higher than sniffing pure urinary signals in both sexes and strains.

4. Discussion

Our results revealed that tested stimuli vary in their signalling potential as strain-specific indicators in the house mouse mate recognition system. The longest time spent by investigation of stimuli and strongest preferences for the same strain were found for urine, faeces, bedding, urine + saliva and urine + ABP, whereas the weakest preferences and interest were found for saliva and ABP presented as single stimuli. We have shown significant differences in levels of assortative preference in both males and females. Based on consideration of higher cost of reproduction for females, we expected to find stronger preferences displayed by this sex than the opposite. However, in our data *M. m. musculus* derived-males displayed the strongest assortative preferences. Weaker preferences found in females even of the same strain could be explained by the fact that sexual receptivity of tested females was not achieved in all tests, but we did not find significant differences in assortative preferences between females in receptive and non-receptive phase of their oestrus cycle. Moreover there is growing evidence that house mice males show stronger assortative preferences than females (Piálek et al., 2008; Ganem et al., 2008). Finally, our data revealed strong differences in assortative preferences of the two strains derived from wild individuals of each house mouse subspecies. Since this study was based on a highly simplified model using olfactory experiments as representation of mate choice tests and two inbred strains, preserving only a small portion of genetic variation present in wild populations of the two house mouse subspecies, we remain cautious in extrapolating the results to wild populations.

Broad surveys of olfactory communication suggest a variety of pheromones affecting behavioural response of the receiver individually, in groups of different odorants or acting in concerns with other signal modalities and in addition largely interact with the environment (Wyatt, 2003; Novotny, 2003; Brennan and Kendrick, 2006; Brennan and Zufall, 2006). The modulation of signals and their complexity is rather a rule in animal communication, as these are important in signal repeatability, modulation of signal intensity by adding signal components or completion of the signal by combining different signal channels (Wyatt, 2003). For example, in *Drosophila* a successful courtship requires combination of chemical and visual stimuli (Greenspan and Ferveur, 2000) or male golden hamsters use several different odours from the same female to form a multicomponent representation of female individuals (Johnston and Bullock, 2001). However, as far as we know, there has been no study in the house mouse using a combination of different stimuli to test whether these act together to reinforce or complete the information transmitted from the donor.

In our study, we directly tested the effect of combining two signal components, previously identified as candidates for subspecies-specific indicators (Smadja et al., 2004; Laukaitis et al., 1997) on resulting signal interest and preference. We expected that the combined signals would convey more complex information and thus would elicit stronger mate choice preference. However, in our study, neither the preferences for combined stimuli (urine + saliva, urine + ABP) was enhanced in comparison with preferences for equal individual compounds nor the longer time spent by investigating the combined, two-compound stimuli was observed. Thus the potential additive or synergistic effects of urine and saliva (including ABP) were not confirmed here. Accordingly, we did not find preferences for beddings, used in this study as a representative of the complex mouse odour, as the strongest preferences in neither sex nor strain even though the time spent by the investigation was significantly higher than for any other stimuli. It indicates that more complex stimuli do not possess more information facilitating mate choice. Conversely, in males of *domesticus*-derived STRA strain we found significant disassortative preference. This result may reflect the fact that bedding was present in a larger amount of stimulus relative to other tested stimuli and/or that bedding was placed in the peripheral boxes that the subject was allowed to enter and, consequently, presented an area similar to the marked territory. Observed preferences, not found in females, thus can be interpreted as strong male avoidance of *domesticus* like territory/odour, that can be caused by high levels of male aggressiveness previously reported in this strain (Piálek et al., 2008) and in *domesticus* males in general (Thuesen, 1977; van Zegeren and van Oortmerssen, 1981; Munclinger and Frynta, 2000; Frynta et al., 2005).

Our study is in agreement with previously published data reporting that of the two subspecies, *M. m. musculus* more often displays homosubspecific preferences and is proposed as a choosier subspecies (Christophe and Baudoin, 1998; Smadja and Ganem, 2002, 2005; Smadja et al., 2004; Bímová et al., 2005; Ganem et al., 2008; Fig. 1), whereas *M. m. domesticus* mice were reported to display homosubspecific preferences only for ABP (Laukaitis et al., 1997; Talley et al., 2001). On the other hand, we found the strongest assortative preferences for faeces in both females and males of the *domesticus*-derived STRA strain. It has been shown that faeces could provide information on an individual's reproductive and/or health status, including stress, age, phase of the oestrus cycle (Schwarzenberger et al., 1996; Touma et al., 2003) and/or parasite load, metabolism and food resources (Kavaliers et al., 2004, 2005). Since all experimental animals (both subjects and 'signal' donors) were parasite-free (based on a regular veterinary diagnosis of the inbred lines in our breeding facility), and kept in standard laboratory conditions, the difference in the discrimination of faeces between the two strains could reflect their differences in immune system (for both strains described in Piálek et al., 2008), microbial composition of intestines that is highly variable in mice (Scupham et al., 2006) or differences in steroid metabolites (Touma et al., 2003). However, as the same pattern was not observed when using soiled bedding we cannot rule out a possibility this result to be accidental.

The central role of urine as a principal olfactory cue in mice (Novotny, 2003; Smadja and Ganem, 2005) is confirmed also in our results. Urine alone elicited the longest investigation (data not shown) and strongest preferences than either of its combinations especially in *musculus*-derived BULS mice. This corroborates the evidence that urinary pheromones (volatiles coded for by MHC-genes or protein complexes of MUPs) are important in modulating individual signalling in different social interactions such as aggression, territory defence and/or sexual behaviour (Novotny, 2003; Thom and Hurst, 2004; Brennan and Kendrick, 2006). Contrary to the genetic relatedness primarily manifested by volatiles coded for by MHC and background genes (Penn, 2002; Willse et al., 2006; Röck et al., 2007), MUPs may present a more reliable individual scent signature (Hurst et al., 2005; Cheetham et al., 2007; Sherborne et al., 2007; Thom et al., 2008) and an additive source of information in their ability to advertise temporal individual-status information such as female oestrus (Stopka et al., 2007), social condition or male competitive ability (Rusu et al., 2008; Rich and Hurst, 1998, 1999; Beynon and Hurst, 2003). Both sexes can, by varying their MUPs expression, modulate their signalling in different social interactions in order to maximize their fitness (Stopka et al., 2007). Thus,

MUPs may present ideal candidates for targets of natural selection and act as subspecies-specific recognition indicators (Beynon et al., 2007).

In fact, recent studies identified significant differences in MUPs expression both between the two subspecies (Stopková et al., 2007) and different mouse species (Robertson et al., 2007). Pronounced sexual dimorphism in MUPs expression was also shown to be subspecies-specific: *M. m. musculus* males expressed MUPs in higher concentration than females of the same subspecies and significantly more than either sex in *M. m. domesticus*. In addition, individual variation in the expression of most studied MUPs was lower than between sexes and subspecies (Stopková et al., 2007). These data together with strong attractiveness of urine of *musculus*-derived BUSNA males for *musculus*-derived BULS females but not for *M. m. domesticus*-derived mice of both sexes reported in our study implies that urinary MUPs may primarily serve to communicate strain-specific and further more subspecies-specific information.

Compared to bedding, faeces and urine, our results revealed much weaker interest in saliva (saliva and ABP) indicating that salivary proteins (such as salivary MUPs and ABP) may not be the strongest subspecies-specific indicators as proposed by Laukaitis et al. (1997), Laukaitis and Karn (2005) and Talley et al. (2001). Our results seem to be corroborated by both direct (Bímová et al., 2005) and indirect (Dod et al., 2005; Macholán et al., in press) evidence from the *musculus/domesticus* hybrid zone, where only slight ABP-specific preferences and nearly neutral transition of the *Abpa* gene across the zone has been noted. However, it may be questioned to what extent our experimental design using simplified olfactory preference tests may reflect mate choice occurring in the natural conditions.

The lowest preferences and investigation time found in the *musculus*-derived BULS strain can be due to possible confounding signalling effect of b-congenic strain (see Bímová et al., 2005), where the *musculus* ABP-type signal is presented together with *domesticus* signals from the *domesticus* genetic background of the C3H/HeJ inbred strain (Frazer et al., 2007). In spite of this, if the ABP should be recognised as subspecies-specific indicator in this experimental design, we should found assortative preferences of *musculus*-derived BULS subjects in tests where saliva from *musculus* inbred strain have been used and of *domesticus*-derived STRA subjects in all tests with salivary stimuli. However, our data do not support this hypothesis. Recent data led to the description of different ABP paralogues that show extensive spatial, temporal and sexual differences in expression (Emes et al., 2004; Laukaitis et al., 2005) mainly in lacrimal and salivary glands located on the head of the mouse. As the first interaction between two mice typically involves investigation either of urogenital or facial, nasal and mouth area (Luo et al., 2003; B. Bímová, unpublished data), we suggest that if ABP is involved in mouse olfactory communication, its role is probably in transmitting information between the animals in close contact. This theory is corroborated by results of ABP-dependent mate choice tests. In experiments, where a direct contact of the tested female with signal males was allowed the assortative choice rate was up to 7:1, whereas in olfactory tests where the female had to make a decision based on olfactory choice of territory or saliva spots the choice rate decreased up to 1.6–2:1 (Laukaitis et al., 1997; Talley et al., 2001). We thus propose that the confirmation of the pheromonal role of ABP (and saliva) as close-contact signals requires a different experimental design allowing contact with animal stimuli and decision made based on complex information of secretions from the head and neck glands. Nonetheless, we maintain that the role of long-lasting signals (such as urine and faeces) can be evaluated based on olfactory experiments, used in this study, simulating a situation where mice are not in direct contact, as occurs frequently in their natural environment (Hurst, 1988, 1990).

Based on results of our study, we have demonstrated that house mice can detect and process a variety of odour signals to get context-dependent information from its environment or consubspecifics. The data strongly suggest that urinary signals are the most obvious candidates for signals involved in individual recognition as well as strain-specific signalling. Moreover both strains use different signals to discriminate consubspecifics (e.g. faeces in *M. m. domesticus* derived strain and urine in *M. m. musculus* derived strain). Males seem to invest more energy in mate choice than females as they show the strongest preferences and invest relatively more in signalling (e.g. higher expression of MUPs profiles in males).

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