

Divergence of odorant signals within and between the two European subspecies of the house mouse

Carole Smadja and Guila Ganem

Laboratoire Génétique et Environnement, Institut des Sciences de l'Evolution de Montpellier, UMR 5554 (UMI/CNRS), Université Montpellier II, 34095 Montpellier Cedex 5, France

The olfactory acuity of mice allows them to discriminate odors of conspecifics differing by a few genes. This acuity is used in habituation procedures where investigation of novel odors by the mouse can be translated into relative difference or similarity between the stimuli. This study adapts these behavioral procedures to address suprapopulation divergence among urinary odors in the house mouse. Specifically, we investigate geographical patterns of odor divergence within and between 2 subspecies of the house mouse, *Mus musculus musculus* and *Mus musculus domesticus*, which diverged in allopatry and met secondarily in Europe where they hybridize. Based on *M. m. musculus* perception, our study suggests that odors of the 2 subspecies differ in both allopatric and contact zone populations and that divergence is more marked in the latter. Our earlier studies documented mate preference and signal divergence between the 2 subspecies. Hence, we consider the role of the urinary odors as mating signals. We discuss how signal divergence between the 2 subspecies may relate to reproductive character displacement. This study validates the use of habituation procedures to reconstruct geographical patterns of odorant signal divergence, providing a strong methodological platform to address reproductive character displacement affecting cryptic mating signals in mammals. *Key words*: habituation–discrimination/generalization, mating signals, odors in urine, olfaction, premating isolation, reproductive character displacement, speciation. [*Behav Ecol* 19:223–230 (2008)]

Variation in mate recognition characteristics is an important factor promoting speciation in many evolutionary lineages (West-Eberhard 1983; Coyne and Orr 1989; Butlin and Ritchie 1994; Lambert and Spencer 1995). When hybridization occurs between parapatric taxa and is detrimental to their fitness, natural selection may directly reinforce divergence of mate recognition systems (Dobzhansky 1940; reviewed in Noor 1999; Servedio and Noor 2003; Servedio 2004). Here we refer to reinforcement as the strengthening of prezygotic isolation in response to selection against hybridization and consider reproductive character displacement (stronger prezygotic divergence in sympatry than in allopatry) as a possible, but not necessary, consequence of reinforcement (Noor 1999).

Theoretically, selection against hybridization is expected to act simultaneously on the signal and the receiver components of a mate recognition system. However, this may not necessarily lead to similar divergence of signals and preferences (i.e., the receiver response) in the contact zone. For instance, Noor (1999) suggested that signals may not diverge if reinforcement acts to narrow the range of signals acceptable to females rather than shifting their mean phenotype. Alternatively, referring to their numerical model, Lemmon et al. (2004) proposed that when selection shifts the mean phenotype of signals and preferences, the 2 components of the mate recognition system could follow qualitatively different patterns of divergence. Indeed, the simulations showed that under low selective pressures, only divergence in mating signals could be detected indicating that, under certain conditions of selec-

tion, signals would tend to diverge more rapidly than preferences. Conversely, strong selection against hybridization would result in divergence of both signals and preferences (Lemmon et al. 2004). Moreover, sexual selection can rapidly increase divergence, presumably through a runaway process, and this force is expected to facilitate coupled divergence of the 2 components of the mate recognition system (Lande 1981; Liou and Price 1994; Higashi et al. 1999; Noor 1999). Consequently, it seems important to study the 2 components of a mate recognition system if one wishes to understand the type and the magnitude of selection acting on these traits.

Studies that have investigated divergence of the 2 components of the mate recognition system in the context of speciation have concerned birds (e.g., Saetre et al. 1997), amphibians (e.g., Höbel and Gerhardt 2003; Hoskin et al. 2005), or insects (e.g., Marshall and Cooley 2000; Jang and Gerhardt 2006) that utilize acoustic or visual mating signals that can be directly observed and investigated through, for example, song recording or colorimetric analyses. Divergence of mating signals based on chemical cues has been investigated more rarely, probably, because these are cryptic signals that may be more difficult to capture. Most studies have concerned insects, whose chemical mating signals, such as cuticular hydrocarbons (e.g., *Drosophila serrata/birchii*; Higgie et al. 2000) or long-range sex pheromones (e.g., the European Corn borer: Roelofs et al. 1987), have relatively simple and identifiable characteristics. In mammals, and particularly in rodents, we know that signaling of individual (Boyse et al. 1987; Penn and Potts 1998; Hurst et al. 2001; Thom and Hurst 2004; Beauchamp and Yamazaki 2005), population, and species (Laukaitis et al. 1997; Christophe and Baudoin 1998; Heth et al. 2001; Smadja and Ganem 2002) identity occurs mainly through odors, suggesting that olfactory communication may play an important role in premating isolation. However, very few studies have addressed sexual isolation based on olfactory cues in mammals (but see Nevo et al.

Address correspondence to C. Smadja, who is now at Department of Animal and Plant Sciences, The University of Sheffield, Western Bank, Sheffield S10 2TN, UK. E-mail: c.smadja@sheffield.ac.uk.

Received 21 March 2007; revised 1 October 2007; accepted 2 November 2007.

1976; Pillay et al. 1995; Theiler and Blanco 1996), and to our knowledge, no study has investigated specifically geographical patterns of odorant signal divergence, possibly because the chemical characteristics of such signals are difficult to analyze (Novotny 2003; Todrank and Heth 2003; Brennan and Keverne 2004; Hurst and Beynon 2004).

The present study addresses signal divergence between 2 subspecies of the house mouse sharing a hybrid zone where there is evidence for selection against hybridization (Britton-Davidian et al. 2005; Dod et al. 2005; Raufaste et al. 2005). *Mus musculus domesticus* and *Mus musculus musculus* diverged genetically during approximately 0.5 million years in allopatry and reestablished contact in Europe approximately 5000 years ago (Boursot et al. 1993). *Mus musculus musculus* and *M. m. domesticus* populations in the border of the hybrid zone were shown to possess distinct subspecific signals, present in the urine, and allowing *M. m. musculus* to recognize a consubspecific and mate assortatively (Smadja and Ganem 2002; Smadja et al. 2004). Moreover, investigations of geographical patterns of mate preference divergence indicated, first, that assortative preference occurred only in the contact zone and, second, that contact populations of *M. m. musculus* displayed stronger assortative preference than contact populations of *M. m. domesticus* (Smadja and Ganem 2005). This earlier study indicated asymmetrical reproductive character displacement of the receiver component of the 2 subspecies' mate recognition systems. The present study concerns the mating signals and aims to test whether they show a geographical pattern of divergence consistent with that evidenced for mate preference.

We applied different habituation procedures to assess suprapopulation divergence among cryptic odorant signals and to reconstruct the pattern of signal divergence between the 2 subspecies. This approach relies on the demonstrated olfactory acuity of mice (Boyse et al. 1987; Isles et al. 2001; Carroll et al. 2002; Brennan 2004) and their use of this acuity in their natural environment (Hurst 1990; Rich and Hurst 1998; Thom and Hurst 2004; Hurst and Beynon 2004). The habituation–discrimination and the habituation–generalization procedures (see a review in Todrank and Heth 2003) assess odor differences and relative similarities from the duration of investigation of odor stimuli by a mouse, hereafter called a “nose” (or a potential “receiver”). Discrimination between odors indicates, without ambiguity, that they differ, whereas absence of discrimination can either indicate odor similarity or that the nose does not perceive the differences. Assessment of relative odor similarities gives insights into the degree of divergence among several signals.

We designed 5 successive experiments, involving habituation–discrimination and habituation–generalization procedures, to compare the extent of divergence between mating signals from mice sampled from contact zone and allopatric populations of each subspecies. The first experiment explored whether the signals of allopatric populations of the 2 subspecies differed. A pair of experiments addressed whether signals of allopatric and contact zone populations have diverged within each subspecies. Finally, a pair of experiments compared relative divergence of signals of allopatric and contact zone populations of the 2 subspecies. It is the combined results of this series of experiments that allowed us to infer the pattern of signal divergence within and between the 2 subspecies and to assess whether this divergence was more marked in contact zone populations than in allopatric populations of the 2 subspecies. Mate preference is known to differ between the 2 subspecies (Smadja and Ganem 2005), further suggesting that the odors involved in divergence evidenced in the present study could be mating signals. The mechanisms that may be involved in this divergence are also discussed with reference to the available theoretical studies.

MATERIAL, GENERAL METHODS, AND CONTROLS

Material

The signals: urinary stimuli

All stimuli were composed of the urine of female mice kept in standardized laboratory conditions. House mouse urine is known to carry information on different aspects of individual and population characteristics (e.g., Christophe and Baudoin 1998; Heth et al. 2001; Hurst et al. 2001). To assess divergence at the geographical and subspecies levels, the stimuli used in this study were pools of urine of several individuals and populations so as to represent the 4 combinations of geographical areas \times subspecies. Pooling the odors of individuals is a way to avoid both between-individual variation caused by environmental factors and between-individual genetic variation. Indeed, it decreases the distinguishability of odors within populations thereby revealing discrimination of signals at the population level (Penn and Potts 1998). Therefore, this protocol is appropriate when one wants to assess signal divergence at the population level or at the suprapopulation level (like in our study, at the geographical level). Nevertheless, it requires that we control for odor consistency across populations of a given subspecies. As far as the contact zone stimuli were concerned, our earlier investigations indicated that signal divergence was consistent in the contact zone across individuals and populations within each subspecies (Smadja et al. 2004). Such information was not available as far as the allopatric stimuli were concerned. Hence, we first tested that the odors present in stimuli obtained by pooling urine from several individuals of each of 2 allopatric populations, randomly sampled in each subspecies, were equally different from a contact zone population stimulus (control test 1, see below). Because the results of the control test indicated that odors of stimuli of 2 allopatric populations differed to the same extent from those of the contact zone population, we proceeded to constitute allopatric stimuli by pooling urine of mice from these 2 allopatric populations within each subspecies. Stimulus “*M. m. musculus* allopatric” was composed of urine samples of mice from Telč (Eastern Czech Republic, see details in Smadja and Ganem 2005) and from the laboratory population MPB (Hungary), whereas stimulus “*M. m. domesticus* allopatric” was composed of urine samples of mice from Lisbon (Portugal) and from the laboratory population BIK (Israel). The 2 laboratory populations were obtained from the wild mice genetic repository (CNRS-UMR 5554, Montpellier, France) (<http://www.isem.cnrs.fr/spip.php?article477>). The stimuli, “*M. m. musculus* contact zone” and “*M. m. domesticus* contact zone” were obtained by pooling urine samples from 2 different populations of the contact zone in Denmark (Jutland): “*M. m. musculus*, contact zone a” = Laasby and Ejstrupholm populations; “*M. m. musculus*, contact zone b” = Framlev and Odder; “*M. m. domesticus*, contact zone a” = Lunderskov and Sonder Bjert; “*M. m. domesticus*, contact zone b” = Sommersted and Fjelstrup (for more details on these populations see Smadja et al. 2004). All urine samples were kept at $-20\text{ }^{\circ}\text{C}$.

Animals

Because our aim was to assess signal variation, we homogenized perception by using the same sample of 10 mice as the nose in all the experiments. We chose *M. m. musculus* male mice from populations in the contact zone as the nose in our tests because they displayed a marked assortative preference further testifying that they could discriminate between odors of the 2 subspecies (Smadja et al. 2004; Smadja and Ganem 2005). All mice were first-generation progeny of a wild trapped contact zone population (Denmark, Laasby, see Smadja et al. 2004), born in captivity, and they were tested as adults (older than 1 month). Mice were kept individually, to stimulate

their motivation, under standardized laboratory conditions (food and water available ad libitum, 12:12 photoperiod; lights on between 7 and 19 h).

The habituation–discrimination and habituation–generalization procedures

The habituation–discrimination (review in Halpin 1986) and the habituation–generalization (Todrank and Heth 2003) procedures are based on the same general principle. Each experiment includes 2 phases: an habituation phase during which a mouse (the nose) is presented with a single odor stimulus to which it becomes familiar (i.e., duration of investigation of the stimulus decreases), immediately followed by a discrimination phase during which the nose is presented simultaneously with a pair of odors for which resemblance to the habituation odor is assessed from the relative time spent investigating the 2 test odors. Whereas the habituation phase is identical in the 2 procedures, the discrimination phase differs: in the habituation–discrimination procedure, one of the 2 odors presented in the discrimination phase is identical to the habituation odor, whereas in the habituation–generalization procedure the 2 odors presented during the discrimination phase are different from the habituation one. Practically, during the discrimination phase, a mouse will naturally investigate the less familiar odor for a longer duration than the more familiar odor (habituation–discrimination) or the odor that resembles most the familiar odor (habituation–generalization). Therefore, we used the habituation–discrimination procedure to assess differences between the habituation and a test odor and the habituation–generalization procedure to assess which of 2 test odors is more similar to the habituation one. These procedures have been validated for several rodent species (Heth and Todrank 2001; Columbelli-Negrel and Gouat 2006; Pillay et al. 2006). Nevertheless, we tested that, in the case of *M. m. musculus*, preference did not interfere with discrimination (see control test 2 below).

In our study, the habituation phase lasted 10 min and the discrimination phase 5 min. The tests were performed in a Plexiglas apparatus consisting of 2 boxes: the start box (18 × 24 × 14 cm) and the test box (36 × 24 × 14 cm) connected by a short tunnel (5 × 10 cm). The mouse was placed in the start box at the beginning of each test, and the stimuli were placed in the test box. The test started when the mouse crossed the perforated door separating the start box from the tunnel, after which its behavior, that is, time spent sniffing or licking a stimulus, was recorded using a Psion Organiser and the Observer software (Noldus et al. 2000). During the 10-min habituation phase, the single urine sample was placed directly at the bottom in the middle of the far end of the test box. Total duration of investigation of the habituation stimulus was always lower than 10 min ($n = 80$; mean ± standard error = 23.05 ± 2.39 s). Once the habituation phase ended, the mouse was confined back in the start box. The 5-min discrimination phase followed immediately after the habituation phase and involved 2 urinary stimuli placed on the floor at the left and right corners of a test box which was different from the one used during the habituation phase. Here too, total investigation time of both stimuli was always lower than 5 min ($n = 80$; 13.64 ± 0.95 s). The left and right position of each stimulus was alternated between tests. All the experiments took place between 8 and 13 h. Each series of experiments was separated by at least 1 week, which was enough to avoid interference between 2 consecutive experiments.

Statistical analyses

All the habituation–discrimination and habituation–generalization experiments were first validated by a clear pattern of

habituation displayed by each individual and assessed statistically (Wilcoxon signed-rank unilateral tests) by comparing the time spent investigating the habituation stimulus during the first 5 min with that spent investigating the same stimulus during the final 5 min of the test (Figures 1 and 2, habituation results). Discrimination was assessed statistically by comparing the time spent investigating one versus the other test stimulus (Wilcoxon signed-rank test). We performed unilateral tests for data obtained with the discrimination procedure and bilateral tests for those obtained with the generalization procedure. We used the same sample of nose in the series of 5 experiments to control for variation in perception. Nevertheless, this experimental setting yielded repeated type measures. Therefore, we controlled the proportion of false positives among the rejected null hypotheses using the Benjamini and Hochberg (1995) procedure of correction (see Garcia 2004; Nakagawa 2004 for a comparison with the sequential Bonferroni procedure). *P* values were recalculated separately among the habituation and the discrimination/generalization tests and noted as *P*^{*} in the result sections. All analyses were performed with Statistica software (StatSoft Inc 2001). The same statistical approach was applied to the control tests.

Control tests

Control 1: validation of pooling urine across allopatric populations

To test for consistency between odors of 2 allopatric populations of a given subspecies, we performed habituation–generalization tests using stimuli of the same subspecies. A “contact zone” odor was the habituation stimulus, and 2 distinct allopatric populations’ odors were the discrimination stimuli. A test was performed for each of the 2 subspecies. The stimuli used in this experiment were pools of urine of several individuals from a single population: for *M. m. musculus*: habituation: Framlev population (contact zone); discrimination: Telč population (allopatry 1) versus MPB population (allopatry 2); for *M. m. domesticus*: habituation: Lunderskov population (contact zone); discrimination: Lisbon population (allopatry 1) versus BIK population (allopatry 2) (Figure 1). The 2 tests showed that the test mice spent an equal time investigating the 2 allopatric stimuli after habituation to a contact signal (Figure 1). Moreover, the time spent investigating each of the 2 allopatric stimuli was significantly higher than that during the last period of habituation, indicating that the allopatric odors were perceived as different from the contact zone odors (Figure 1 and statistical results: *M. m. musculus*: habituation vs. test odor 1: $T = 0$, $Z = 2.66$, $P = 0.004 < P^* = 0.0125$; habituation vs. test odor 2: $T = 2$, $Z = 2.60$, $P = 0.0045 < P^* = 0.025$; *M. m. domesticus*: habituation vs. test odor 1: $T = 5.5$, $Z = 2.24$, $P = 0.01 < P^* = 0.0375$; habituation vs. test odor 2: $T = 5.5$, $Z = 2.24$, $P = 0.01 < P^* = 0.05$). Because neither of the 2 allopatric odors was assessed as more similar to the contact zone odor, we pooled the urine samples of the 2 populations of a given subspecies in order to obtain an “allopatric” stimulus (see methods above).

Control 2

A question that may rise when assessing relative investigation of a pair of odors is whether preference could interfere with discrimination and bias the discrimination results. In order to test for the latter, we habituated male *M. m. musculus* from the contact zone (the nose in our study) to the odor that they preferred (i.e., *M. m. musculus* contact zone, Smadja et al., 2004) and tested them with a pair of stimuli comprising the preferred and a *M. m. domesticus* contact zone odor. If preference biased the discrimination procedure, we had

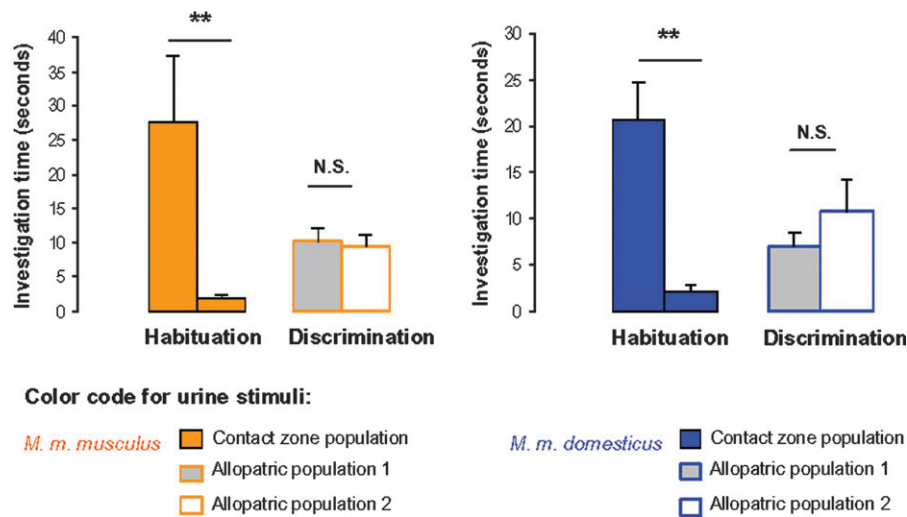


Figure 1

Results of control 1: a validation of pooling urine from 2 allopatric populations within each subspecies. The 2 graphs report results of the 2 control experiments. Results of the habituation (10 min) and the discrimination (5 min) phases are provided for each control test (N.S.: $P > 0.05$; $**P < 0.01$ after Benjamini and Hochberg correction). The first 2 bars of each histogram represent duration of investigation of the habituation stimulus during 1) the first 5 min and 2) the final 5 min of the test. The other 2 bars of each histogram show the time spent investigating each of the 2 test stimuli during the discrimination phase. Tests of effectiveness of habituation: for *Mus musculus* stimuli: $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.025$; for *Mus musculus domesticus* stimuli: $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.05$. Discrimination results: for *M. m. musculus* stimuli: $T = 16$, $Z = 0.28$, $P = 0.78 > P^* = 0.05$; for *M. m. domesticus* stimuli: $T = 14$, $Z = 1.01$, $P = 0.31 > P^* = 0.025$. P^* = corrected level of significance using Benjamini and Hochberg procedure.

2 predictions: either the 2 test odors would be equally investigated or the preferred odor, which was also the habituation odor, would be the one more investigated. Our results show that the most investigated odor, *M. m. domesticus* contact, was not the preferred one (habituation result: $n = 10$, $T = 0$, $Z = 2.80$, $P = 0.0025$; discrimination result: $n = 10$, $T = 0$, $Z = 2.66$, $P = 0.004$). This test allowed us to assert that preference did not interfere with the habituation–discrimination procedure as far as this nose was concerned.

EXPERIMENTS AND RESULTS

Is subspecific divergence of mating signals also found in allopatric populations?

Our previous study investigating preference indicated that odors of contact zone populations of the 2 subspecies differed significantly (Smadja et al. 2004). In order to check whether this divergence occurred in populations distant from the contact zone, and so may have predated the contact, we tested the hypothesis that allopatric stimuli of the 2 subspecies carried distinct urinary odors. This experiment involved the habituation–discrimination procedure. The mice were habituated to the “*M. m. musculus* allopatry” signal, and their ability to discriminate between “*M. m. musculus* allopatry” and “*M. m. domesticus* allopatry” was tested (Figure 2A). We expected the 2 test stimuli to be investigated for the same amount of time if they were assessed as similar by the nose (null hypothesis). Alternatively, we expected the *M. m. domesticus* allopatry signal to be investigated for longer if the allopatric odors of the 2 subspecies were perceived as different.

Our results indicate that the 2 allopatric type signals were assessed as different (Figure 2A), suggesting that the urinary odors of the 2 subspecies differ in allopatry.

Do mating signals of allopatric and contact zone populations of a given subspecies differ?

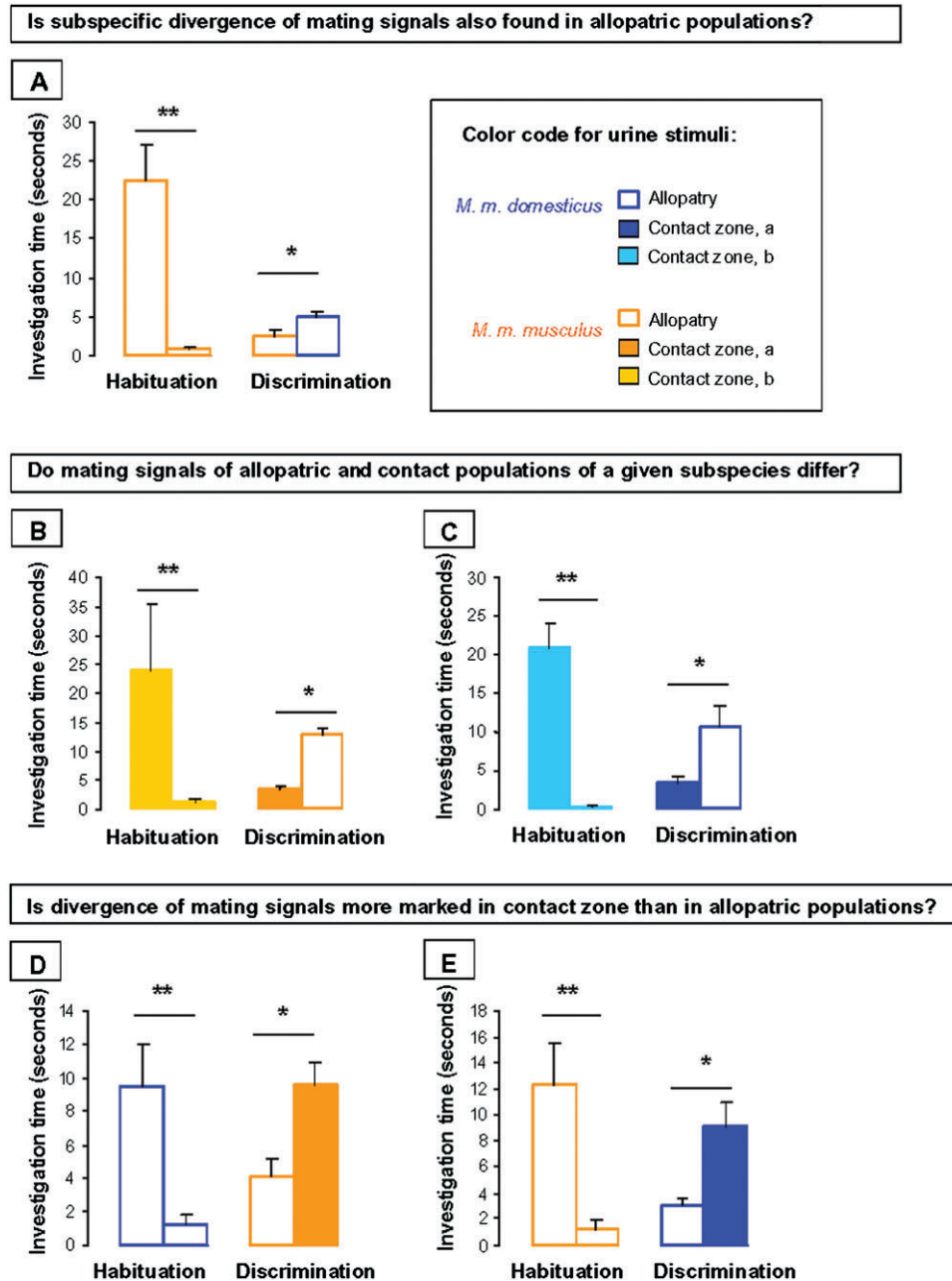
We found evidence for odor divergence between the 2 subspecies both in contact zone populations (Smadja et al. 2004)

and in allopatric ones (this study). However, the extent of subspecies divergence in the 2 geographical areas may differ if populations in the 2 areas experience, for example, different environmental conditions that could translate into different regimes of genetic drift or natural and sexual selection. Allopatric and contact zone populations of a given subspecies would not be expected to present different odors at the suprapopulation level if similar conditions prevail in the 2 geographical contexts. Alternatively, if conditions differ, odors of allopatric and contact zone populations of a given subspecies may be different. To test whether divergence occurred between allopatric and contact zone populations and if this was so for both subspecies, a pair of experiments compared allopatric and contact zone odors within each subspecies (Figure 2B,C). We used the habituation–generalization procedure and presented different contact zone stimuli (contact zone a vs. contact zone b) during habituation and discrimination so as to compare the extent of divergence between 2 contact zone odors of a given subspecies versus a contact zone and the allopatric odors. We expected the 2 test stimuli to be investigated to the same extent if the 3 sets of odors were assessed as equally different (i.e., the null hypothesis). Alternatively, we expected either the allopatric stimulus to be less investigated than the contact zone stimulus or the allopatric stimulus to be more investigated if divergence between contact zone and allopatric populations was greater than that between odors of 2 contact zone populations of the same subspecies.

Our results indicate that the 2 contact zone odors were assessed as more similar to each other than to the allopatric odor, and this was true within both subspecies (Figure 2B,C). This suggests that female odors differ between allopatric and contact zone populations of mice of both subspecies.

Is divergence of mating signals more marked in contact zone than in allopatric populations?

The previous experiments compared the 2 geographical sets of odors within the same subspecies, which does not tell us whether

**Figure 2**

Results of the 5 experiments designed to assess patterns of divergence of odorant signals within and between the 2 subspecies of the house mouse. The 5 graphs (A–E) report results of the 5 experiments designed to answer 3 questions. As in Figure 1, results of the habituation (10 min) and the discrimination (5 min) phases are provided for each of the 5 experiments ($*P < 0.05$; $**P < 0.01$ after Benjamini and Hochberg correction). $n = 10$ for all experiments. Tests of effectiveness of habituation: (A) $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.01$; (B) $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.02$; (C) $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.03$; (D) $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.04$; (E): $T = 0$, $Z = 2.80$, $P = 0.0025 < P^* = 0.05$. Discrimination results: (A) $T = 8$, $Z = 1.98$, $P = 0.023 < P^* = 0.04$; (B) $T = 0$, $Z = 2.80$, $P = 0.005 < P^* = 0.01$; (C) $T = 0$, $Z = 2.52$, $P = 0.011 < P^* = 0.03$; (D) $T = 6$, $Z = 1.95$, $P = 0.048 < P^* = 0.05$; (E) $T = 0$, $Z = 2.66$, $P = 0.008 < P^* = 0.02$. P^* = corrected level of significance using Benjamini and Hochberg procedure.

odors of the 2 subspecies differ more, or less, in one or the other geographical context. The habituation–generalization procedure allows us to compare 3 odors, whereas a direct assessment of the extent of divergence between odors of the 2 subspecies in the contact zone and in allopatry involves comparing 4 odors. Hence, we had to perform 2 symmetric experiments in which the habituation stimulus was an “allopatric” odor of one of the subspecies and the 2 discrimination stimuli were an “allopatric” and a “contact zone” odor of the other subspecies (Figure 2D,E). When the *M. m. domesticus* allopatric stimulus was used as the habituation odor, we expected stimuli *M. m. musculus* contact zone and *M. m. musculus* allopatric to be equally investigated during the discrimination phase if they were perceived as equally different from the habituation odor (null hypothesis). Alternatively, greater investigation of the *M. m. musculus* contact zone stimulus was expected if divergence was perceived as more marked in the contact zone than in

allopatry among *M. m. musculus* stimuli or the other way round if divergence of signals in the contact zone was less marked.

The results indicate that, although they pertained to different subspecies, the allopatric stimuli were assessed as more similar to each other than to a contact zone stimulus (Figure 2D,E), and this was consistent in the 2 subspecies. Moreover, by deduction, a lower divergence between allopatric signals than between allopatric and contact zone signals of the 2 taxa strongly suggests that this divergence between subspecies is more marked in the contact zone than in allopatry.

DISCUSSION

The behavioral approach adopted in this study demonstrated divergence between allopatric signals of the 2 subspecies and differences between allopatric and contact zone signals within

each subspecies. Further, the results suggest that divergence is more marked between the 2 subspecies signals in contact zone than in allopatric populations.

Obviously, a mouse cannot discriminate between identical signals. However, assessment of relative divergence between signals may be attributed to the specific perception characteristics of a given nose as such assessment may rely on cognitive handling of the task of comparing 3 different odors, a process which may vary between noses. In our study, perception of signal divergence was assessed only in male *M. m. musculus*, chosen as the nose for their previously demonstrated higher ability to discriminate between odors of the 2 subspecies than *M. m. domesticus* mice (Smadja et al. 2004; Smadja and Ganem 2005). The question of whether the 2 subspecies would perceive this divergence with the same acuity still needs to be investigated and should provide important insights concerning the mechanisms involved in the behavioral divergence between them. Nevertheless, whereas perception of divergence relates to the nose used in this study, failure to detect divergence by another nose would indicate differences in the perception characteristics of the 2 noses but would not contradict the fact that signal divergence has occurred.

Geographical proximity between populations may explain greater similarity between 2 nearby contact zone stimuli (contact a and contact b) as compared with the more distant allopatric stimulus (Figure 2B,C). Nevertheless, the perception of odor similarity did not seem to relate to geographical distance consistently across our study. For instance, in another pair of experiments (Figure 2D,E), an allopatric stimulus of 1 subspecies was perceived as more similar to an allopatric stimulus of the other subspecies than to a contact stimulus, although being more geographically distant. Our results suggest that the influence of geography may not relate to actual distances between populations but to whether the populations of the 2 subspecies are close to the contact zone or not.

Assessment of odor variation by *M. m. musculus* males reveals consistent patterns of signal divergence in females of the 2 subspecies. These patterns of signal divergence suggest that the divergence between the 2 subspecies previously observed in the contact zone (Smadja et al. 2004) also exists in allopatric populations but that it is more marked in contact zone populations. Nevertheless, to demonstrate reproductive character displacement requires that a significant component of the among-population variation is explained by the contact with the other taxon, rather than by other factors such as random variation (Butlin 1989, 1995; Gabor and Ryan 2001). A way to control for random variation is to increase the number of populations studied. In the contact zone, an earlier multipopulation-based study indicated that between subspecies signal divergence was consistent across populations of the house mouse in Denmark (Smadja et al. 2004). In allopatry, a more detailed analysis of signal variation across populations of each subspecies is still to be done. Nevertheless, random variation is unlikely to explain our results given the particular patterns of consistent divergence across subspecies revealed here. Although experiments with more combinations of population stimuli would be necessary to confirm a pattern of reproductive character displacement affecting odorant signals between the 2 subspecies of mice (Gabor and Ryan 2001; Jang and Gerhardt 2006), our study provides the first evidence for divergence of odors between Danish populations of the contact zone and allopatric populations, within and between the 2 taxa. Moreover, the most plausible interpretation is that these odors have a function in mate recognition.

By documenting for the first time geographical divergence in odorant signals among contact zone and allopatric populations of 2 hybridizing rodent taxa, our study shows that habituation procedures can help to assess suprapopulation

divergence among cryptic odorant signals, thus providing a strong methodological platform to further studies addressing the occurrence of reproductive character displacement in odorant-based mate recognition systems.

Most studies that have evidenced reproductive character displacement relied on analyses of either mating signals (e.g., Otte and Endler 1989; Benedix and Howard 1991; Loftus-Hills and Littlejohn 1992) or mate preferences (e.g., Gerhardt 1994; Noor 1995; Ryan et al. 1996; Rundle and Schluter 1998; Pfennig et al. 2000; Gabor and Ryan 2001; Nosil et al. 2003) or investigated divergence of mate preferences and mating signals in only one of the 2 hybridizing taxa (Saetre et al. 1997; Higgie et al. 2000; Höbel and Gerhardt 2003). Our work on the house mouse concerns mate preference (Smadja and Ganem 2005) and recognition signals (this study) and suggests that enhanced divergence in the contact zone, as compared with allopatry, involves both subspecies and may be present in both mate preferences and mating signals.

Combined information on preferences and signals may be helpful in distinguishing the mechanisms underlying the evolution of these traits (Noor 1999). As proposed by Noor, enhanced divergence of a mate recognition system in sympatry can result from very different mechanisms: differential fusion, signal interference, physical environmental conditions, and selection against hybridization. In the house mouse, there is evidence for no mechanism other than selection against hybridization (Raufaste et al. 2005), suggesting that this form of selection is the most likely cause of reproductive character displacement between the 2 subspecies (Smadja and Ganem 2005). Reinforcement in sympatry has been shown to be facilitated by initial divergence in allopatry, for example, via differential regimes of drift or natural or sexual selection (Liou and Price 1994; Rice and Pfennig 2006). By showing that divergence of mating signals may occur in allopatric populations, our results suggest that favorable conditions may have prevailed before the contact for reinforcement to occur. In this context, the patterns of signal divergence revealed in this study suggest that selection might have proceeded through a shift of the mean phenotype, rather than by reducing the range of signals acceptable to sympatric individuals. This qualitatively similar pattern of divergence of mate preferences and mating signals suggests that the 2 components of the recognition system have followed parallel evolutionary paths. As suggested by models of reinforcement, this coupled divergence of both signals and preferences is expected only under strong regimes of selection against hybridization (Lemmon et al. 2004), which may be consistent with the magnitude of selection estimated in the hybrid zone (Raufaste et al. 2005).

In conclusion, our study provides the first qualitative picture of divergence of urinary signals within and between the 2 subspecies. To confirm and go further in understanding the mechanisms underlying divergence of the mate recognition system between the 2 subspecies of the house mouse, further studies should address the chemical characteristics underlying signal divergence and its genetic basis. A preliminary study suggests that the molecules involved in subspecific signaling may have low volatility (Ganem et al. 2005). A candidate family of molecules to investigate could be the major urinary proteins, which have been shown to play a role in individual recognition (Bacchini et al. 1992; Brennan 2001; Hurst et al. 2001) and to vary between house mice populations (Payne et al. 2003) and between mouse species (Robertson et al. 2007).

FUNDING

CNRS-UMR 5554; French Ministry of Research; European Community (Marie Curie Individual Fellowship to C.S.); UMR 5554 (UMII/CNRS) (ISEM 2007-085).

We thank J. Britton-Davidian, T. Lenormand, T.J. Robinson, R.K. Butlin, J. Todrank-Heth, and E.B. Keverne for comments, discussions, and suggestions on earlier versions of the manuscript. We also thank the 2 anonymous reviewers for constructive comments that improved our article. Special thanks to A.C. Nunes Oliveira for helping to adapt the technique of habituation-generalization and M. Sanguinet-Perriat for looking after our mice.

REFERENCES

- Bacchini A, Gaetani E, Cavaggioni A. 1992. Pheromone binding proteins of the mouse, *Mus musculus*. *Experientia*. 48:419–421.
- Beauchamp GK, Yamazaki K. 2005. Individual differences and the chemical senses. *Chem Senses*. 30:i6–i9.
- Benedix JH, Howard DJ. 1991. Calling song displacement in a zone of overlap and hybridization. *Evolution*. 45(8):1751–1759.
- Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J Roy Statist Soc Ser B-Meth*. 57:289–300.
- Boursot P, Auffray J-C, Britton-Davidian J, Bonhomme F. 1993. The evolution of house mice. *Ann Rev Ecol Evol Sys*. 24:119–152.
- Boyse EA, Beauchamp GK, Yamazaki K. 1987. The genetics of body scent. *Trends Genet*. 3:97–102.
- Brennan PA. 2001. How mice make their mark. *Nature*. 414:590–591.
- Brennan PA. 2004. The nose knows who's who: chemosensory individuality and mate recognition in mice. *Horm Behav*. 46:231–240.
- Brennan PA, Keverne EB. 2004. Something in the air? New insights into mammalian pheromones. *Curr Biol*. 14:R81–R89.
- Britton-Davidian J, Fel-Clair F, Lopez J, Alibert P, Boursot P. 2005. Postzygotic isolation between the two European subspecies of the house mouse: estimates from fertility patterns in wild and laboratory-bred hybrids. *Biol J Linn Soc*. 84(3):379–393.
- Butlin RK. 1989. Reinforcement of premating isolation. In: Otte D, Endler JA, editors. *Speciation and its consequences*. Sunderland (MA): Sinauer Associates, Inc. p. 158–179.
- Butlin RK. 1995. Reinforcement: an idea evolving. *Trends Ecol Evol*. 10:432–434.
- Butlin RK, Ritchie MG. 1994. Mating behaviour and speciation. In: Slater PJB, Halliday TR, editors. *Behaviour and evolution*. Cambridge (UK): Cambridge University Press. p. 43–79.
- Carroll SP, Penn DJ, Potts WK. 2002. Discrimination of MHC-derived odors by untrained mice is consistent with divergence in peptide-binding region residues. *Proc Natl Acad Sci USA*. 99:2187–2192.
- Christophe N, Baudoin C. 1998. Olfactory preferences in two subspecies of mice *Mus musculus musculus* and *Mus musculus domesticus* and their hybrids. *Anim Behav*. 56(2):365–369.
- Columbelli-Negrel D, Gouat P. 2006. Male and female mound-building mice, *Mus spicilegus*, discriminate dietary and individual odours of conspecifics. *Anim Behav*. 72:577–583.
- Coyne JA, Orr HA. 1989. Patterns of speciation in *Drosophila*. *Evolution*. 43:362–381.
- Dobzhansky T. 1940. Speciation as a stage in evolutionary divergence. *Am Nat*. 74:312–321.
- Dod B, Smadja C, Karn RC, Boursot P. 2005. Testing for selection on the Androgen-Binding-Protein in the Danish house mouse hybrid zone. *Biol J Linn Soc*. 84(3):447–459.
- Gabor CR, Ryan MJ. 2001. Geographical variation in reproductive character displacement in mate choice by male sailfin mollies. *Proc R Soc Lond B Biol Sci*. 268:1063–1070.
- Ganem G, Ginane C, Ostrowski M-F, Orth A. 2005. Assessment of mate preference in the house mouse with reference to investigations on assortative mating. *Biol J Linn Soc*. 84:461–471.
- Garcia LV. 2004. Escaping the Bonferroni iron claw in ecological studies. *Oikos*. 105:657–663.
- Gerhardt HC. 1994. Reproductive character displacement of the grey treefrog *Hyla chrysoscelis*. *Anim Behav*. 47:959–969.
- Halpin ZT. 1986. Individual odors among mammals: origins and functions. *Adv Study Behav*. 16:39–70.
- Heth G, Todrank J, Busquet N, Baudoin C. 2001. Odour-genes covariance and differential investigation of individual odours in the *Mus* species complex. *Biol J Linn Soc*. 73:213–220.
- Higashi M, Takimoto G, Yamamura N. 1999. Sympatric speciation by sexual selection. *Nature*. 402:523–526.
- Higgie M, Chenoweth S, Blows MW. 2000. Natural selection and the reinforcement of mate recognition. *Science*. 290:519–521.
- Höbel G, Gerhardt HC. 2003. Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). *Evolution*. 57(4):894–904.
- Hoskin CJ, Higgie M, McDonald KR, Moritz C. 2005. Reinforcement drives rapid allopatric speciation. *Nature*. 437:1353–1356.
- Hurst JL. 1990. Urine marking in populations of wild house mice *Mus domesticus* Ruddy. III. communication between the sexes. *Anim Behav*. 40:233–243.
- Hurst JL, Beynon RJ. 2004. Scent wars: the chemobiology of competitive signalling in mice. *Bioessays*. 26:1288–1298.
- Hurst JL, Payne CE, Nevison CM, Marie AD, Humphries RE, Robertson DHL, Cavaggioni A, Beynon RJ. 2001. Individual recognition in mice mediated by major urinary proteins. *Nature*. 414:631–634.
- Isles AR, Baum MJ, Ma D, Keverne EB, Allen ND. 2001. Urinary odour preferences in mice. *Nature*. 409:783–784.
- Jang Y, Gerhardt HC. 2006. Divergence in the calling songs between sympatric and allopatric populations of the southern wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae). *J Evol Biol*. 19:459–472.
- Lambert DM, Spencer HG. 1995. *Speciation and the recognition concept. Theory and application*. London: The Johns Hopkins University Press.
- Lande R. 1981. Models of speciation by sexual selection on polygenic traits. *Proc Natl Acad Sci USA*. 78:3721–3725.
- Laukaitis CM, Crister ES, Karn RC. 1997. Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus*. *Evolution*. 51:2000–2005.
- Lemmon AR, Smadja C, Kirkpatrick M. 2004. Reproductive character displacement is not the only possible outcome of reinforcement. *J Evol Biol*. 17(1):177–183.
- Liou LW, Price TD. 1994. Speciation by reinforcement of premating isolation. *Evolution*. 48:1451–1459.
- Loftus-Hills J, Littlejohn MJ. 1992. Reinforcement and reproductive character displacement in *Gastrophysne carolinensis* and *G. olivacea* (Anura: Microhylidae): a reexamination. *Evolution*. 46:896–906.
- Marshall DC, Cooley JR. 2000. Reproductive character displacement and speciation in periodical cicadas, with description of a new species, 13-year *Magicicadas neotredicim*. *Evolution*. 54:1313–1325.
- Nakagawa S. 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behav Ecol*. 15:1044–1045.
- Nevo E, Bodmer M, Heth G. 1976. Olfactory discrimination as an isolating mechanism in speciating mole rats. *Experientia*. 32:1511–1512.
- Noldus LPJJ, Trienes RJH, Hendriksen AHM, Jansen H, Jansen RG. 2000. The observer video-pro: new software for the collection, management, and presentation of time-structured data from videotapes and digital media files. *Behav Res Methods Instrum Comput*. 32(1):197–206.
- Noor MAF. 1995. Speciation driven by natural selection in *Drosophila*. *Nature*. 375:674–675.
- Noor MAF. 1999. Reinforcement and other consequences of sympatry. *Heredity*. 83:503–508.
- Nosil P, Crespi BJ, Sandoval CP. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. *Proc R Soc Lond B Biol Sci*. 270:1911–1918.
- Novotny MV. 2003. Pheromones, binding proteins and receptor responses in rodents. *Biochem Soc Trans*. 31:117–122.
- Otte D, Endler JA. 1989. *Speciation and its consequences*. Sunderland (MA): Sinauer Associate, Inc.
- Payne CE, Malone N, Humphries RE, Hurst JL. 2003. Heterogeneity of major urinary proteins in house mice: population and sex differences. *Chemical signals in vertebrates*. 9:233–240.
- Penn D, Potts WK. 1998. Untrained mice discriminate MHC-determined odors. *Physiol Behav*. 63:235–243.
- Pfennig KS, Rapa K, McNatt R. 2000. Evolution of male mating behavior: male spadefoot toads preferentially associate with conspecific males. *Behav Ecol Sociobiol*. 48:69–74.
- Pillay N, Eborall J, Ganem G. 2006. Divergence of mate recognition in the African striped mouse (*Rhabdomys*). *Behav Ecol*. 17:757–764.
- Pillay N, Willan K, Meester J, Cooke J. 1995. Evidence of pre-mating isolation in two allopatric populations of the Vlei rat, *Otomys irroratus*. *Ethology*. 100:61–71.

- Raufaste N, Orth A, Belkhir K, Senet D, Smadja C, Baird SJE, Bonhomme F, Dod B, Boursot P. 2005. Inference of selection and migration in the Danish house mouse hybrid zone. *Biol J Linn Soc.* 84(3):593–616.
- Rice AM, Pfennig DW. 2007. Character displacement: in situ evolution of novel phenotypes or sorting of pre-existing variation? *J Evol Biol.* 20:448–459.
- Rich TJ, Hurst JL. 1998. Scent marks as reliable signals of the competitive ability of mates. *Anim Behav.* 56(3):727–735.
- Robertson DHL, Hurst JL, Searle JB, Gündüz I, Beynon RJ. 2007. Characterization and comparison of major urinary proteins from the house mouse, *Mus musculus domesticus*, and the aboriginal mouse, *Mus macedonicus*. *J Chem Ecol.* 33:613–630.
- Roelofs W, Glover T, Tang XH, Sreng I, Robbins P, Eckenrode C, Lofstedt C, Hansson BS, Bengtsson BO. 1987. Sex-pheromone production and perception in European corn-borer moths is determined by both autosomal and sex-linked genes. *Proc Natl Acad Sci USA.* 84:7585–7589.
- Rundle HD, Schluter D. 1998. Reinforcement of stickleback mate preferences: sympatry breeds contempt. *Evolution.* 52:200–208.
- Ryan MJ, Dries LA, Batra P, Hillis DM. 1996. Male mate preferences in a gynogenetic species complex of Amazon mollies. *Anim Behav.* 52:1225–1236.
- Saetre G-P, Moum T, Bures S, Kral M, Adamjan M, Moreno J. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. *Nature.* 387:589–592.
- Servedio MR. 2004. The what and why of research on reinforcement. *PLoS Biol.* 2:e420.
- Servedio MR, Noor MAF. 2003. The role of reinforcement in speciation: theory and data. *Annu Rev Ecol Evol Syst.* 34:339–364.
- Smadja C, Catalan J, Ganem G. 2004. Strong premating divergence in a unimodal hybrid zone between two subspecies of the house mouse. *J Evol Biol.* 17(1):165–176.
- Smadja C, Ganem G. 2002. Subspecies recognition in the house mouse: a study of two populations from the border of a hybrid zone. *Behav Ecol.* 13:312–320.
- Smadja C, Ganem G. 2005. Asymmetrical reproductive character displacement in the house mouse. *J Evol Biol.* 18(6):1485–1493.
- StatSoft Inc. 2001. Statistica: data analysis software system, Tulsa.
- Symonds MRE, Elgar MA. 2004. Species overlap, speciation and the evolution of aggregation pheromones in bark beetles. *Ecol Lett.* 7(3):202–212.
- Theiler GR, Blanco A. 1996. Patterns of evolution in *Graomys griseo-flavus* (Rodentia, Muridae). III. Olfactory discrimination as a premating isolation mechanism between cytotypes. *J Exp Zool.* 274:346–350.
- Thom MD, Hurst JL. 2004. Individual recognition by scent. *Ann Zool Fenn.* 41(6):765–787.
- Todrank J, Heth G. 2003. Odor-genes covariance and genetic relatedness assessments: rethinking odor-based “recognition” mechanisms in rodents. *Adv Study Behav.* 32:77–130.
- West-Eberhard MJ. 1983. Sexual selection, social competition, and speciation. *Q Rev Biol.* 58:155–183.