Current Biology 17, 1771–1777, October 23, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.10.007

Report

The Genetic Basis of Individual-Recognition Signals in the Mouse

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Summary

The major histocompatibility complex (MHC) is widely assumed to be a primary determinant of individual-recognition scents in many vertebrates [1–6], but there has been no functional test of this in animals with normal levels of genetic variation. Mice have evolved another polygenic and highly polymorphic set of proteins for scent communication, the major urinary proteins (MUPs) [7–12], which may provide a more reliable identity signature ([13, 14] and A.L. Sherborne, M.D.T., S. Paterson, F.J., W.E.R.O., P. Stockley, R.J.B., and J.L.H., unpublished data). We used female preference for males that countermark competitor male scents [15–17] to test the ability of wild-derived mice to recognize individual males differing in MHC or MUP type on a variable genetic background. Differences in MHC type were not used for individual recognition. Instead, recognition depended on a difference in MUP type, regardless of other genetic differences between individuals. Recognition also required scent contact, consistent with detection of involatile components through the vomeronasal system [6, 18]. Other differences in individual scent stimulated investigation but did not result in individual recognition. Contrary to untested assumptions of a vertebrate-wide mechanism based largely on MHC variation, mice use a species-specific [12] individual identity signature that can be recognized reliably despite the complex internal and external factors that influence scents [2]. Specific signals for genetic identity recognition in other species now need to be investigated.

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Results

To establish the genetic basis of scents underlying individual recognition, we focused on two requirements that have not previously been addressed. First, animals must recognize genetic differences between individuals within the context of the normal genetic heterogeneity and behavior of the species concerned. Therefore, we used wild-derived outbred house mice (*Mus musculus domesticus*) rather than genetically homogeneous inbred strains of laboratory mice that derive from only a very small pool of founders and have very abnormal genetic backgrounds and social experience [19, 20]. Second, tests must demonstrate that an individual's identity has been recognized (a perceptual process). Traditional habituation or training tests demonstrate only that a difference between scents has been discriminated with unknown meaning, if any, to the animals concerned [21]. To demonstrate perceptual recognition of individual identity, we need to demonstrate in behavior toward equivalent individuals a predictable functional difference that will only be shown if individuals are recognized [21, 22]. In this study, we make use of female preference for males whose scent marks they have previously encountered, because a male's scent marks advertise his dominance and territory ownership [14, 23–25]. We create an illusion of a status difference between two equivalent individual males, an illusion that causes test females subsequently to be more attracted toward one of the two males, demonstrating that they recognize which individual is which. Under natural conditions, such attraction may provide females with the direct benefits of a well-defended male territory and/or indirect benefits of good genes for their offspring if successful territory owners are preferred as mates.

Contact with Involatile Scent Components

We first tested whether females recognize familiar versus unfamiliar individual males on the basis of airborne volatiles alone or whether they require contact with involatile scent components. As a test of recognition, we used the preference shown by females for males whose scent marks they have previously encountered relative to males whose scent is unfamiliar [23–25]. Female mice were presented with urine streaks from an unrelated male (the identity-learning phase) and then given a choice between the scent owner and an equivalent unfamiliar male placed behind mesh barriers that allowed some physical contact (the recognition phase; see Experimental Procedures). We measured three distinct behavioral responses (see Movies S1–S3 in the Supplemental Data available with this article online). Time spent sniffing through the barrier reflects the need to gather information from the scent source (usually most prolonged toward novel scents) but gives no indication of the *meaning* of any information gained [21, 22]. *Attraction*, indicating whether females were able to correctly identify individual males from the information gained,

was reflected by two behaviors: gnawing and pulling at the barrier to gain access to the male (an unambiguous measure of attraction), and time spent in close proximity but not interacting with the male.

As predicted, after contact with urine marks from one of the two males, females correctly identified and were more attracted to the familiar-scent owner. This was evident both from more prolonged gnawing or pulling at the barrier in front of the familiar-scent owner and from more time in close proximity to but not interacting with the familiar male (Figure 1A). Sniffing tended to be biased toward the unfamiliar male (Figure 1A); females predictably spent more time gathering information from the novel scent. We then tested whether females were able to recognize a familiar-scent owner after exposure only to airborne volatiles from male urine during the learning phase (see Experimental Procedures). Without full contact with a male's scent, females failed to recognize the familiar-scent owner, and the expected preference for the familiar male disappeared. They spent no more time trying to get through the barrier or remain in proximity to the familiar-scent male than to the unfamiliar male. Both males were also investigated equally (Figure 1B).

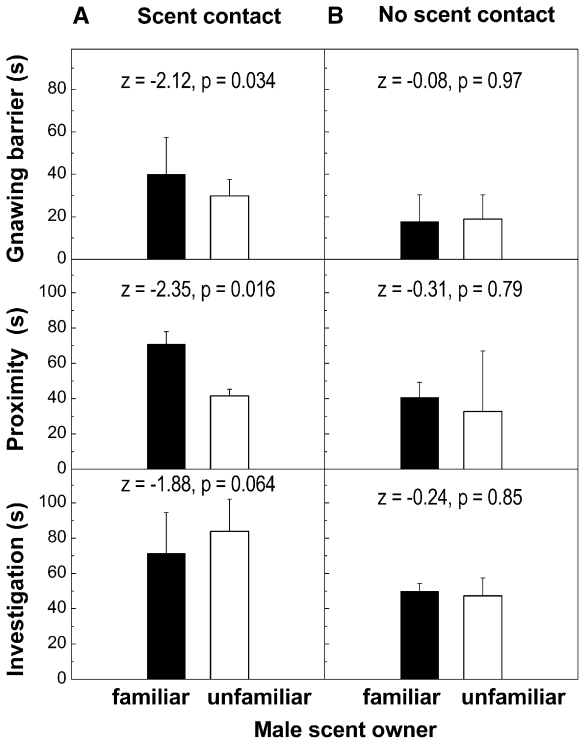

Figure 1. Recognition of Familiar Males with or without Scent Contact

Female recognition of familiar (solid bars) and unfamiliar (open bars) males behind mesh barriers depended on whether they had previous full contact with the familiar male's urine scent or only with airborne volatiles.

(A) After full scent contact, female attraction to the familiar male was clearly evidenced by more prolonged gnawing or pulling at the mesh barrier to gain access to the male. Females also spent more time in proximity to the familiar male without interaction. As expected, investigation behavior (sniffing) tended to be directed more toward the unfamiliar scent male.

(B) Without prior contact with nonvolatile scent cues, females failed to show the expected preference for a familiar male, demonstrating that nonvolatiles are essential for the recognition of the familiar male.

Data are median durations + 75% range, compared via Wilcoxon matched-pairs tests with exact probabilities.

Individual Recognition

To extend our assay from recognition of familiar versus unfamiliar males to a test of individual recognition, we used the well-established preference of females for males that have countermarked a competitor's scent [15–17]. Some species discriminate countermarks deposited on top of, or partially overlapping, a bottom scent [15], but house mice recognize urine countermarks as the freshest, most recently deposited scent [16]. To assess female recognition of individual males when both scents were familiar, we allowed females contact with urine from two males to simulate fresh scent marks from one male that countermarked 24-hr-aged scent marks from the other male (female mice can recognize males from fresh or 24-hr-aged urine marks compared to an unfamiliar-scent owner in similar tests [S.A.C., R.J.B., and J.L.H., unpublished data]). Preference between the two familiar-scent owners was tested after 15 min scent exposure. There was greater attraction (gnawing and proximity) to the owner of the fresh countermarks when males were unrelated to each other and differed in major histocompatibility complex (MHC), major urinary proteins (MUPs), and genetic background, indicating that females could recognize the individual males (Figure 2A). The owner of the aged scent marks induced more sniffing investigation (Figure 2A), suggesting that females initially gained less information from the aged scent. However, as discussed above, a difference in sniffing signifies only that females discriminated a difference in scent between the males that stimulated them to gather further information. This provides no evidence that mice recognized the individual identity of the owner.

Individual Recognition Using MHC Type

To assess whether MHC type was used to recognize individual males, we presented females with scents from two captive-bred males that were full sibs (unrelated to the female) and had inherited either the same MHC type (both haplotypes shared between the males) or a different MHC type (one or both haplotypes differed between the males) but on variable genetic backgrounds. If females can recognize individual males, they should be more strongly attracted to the countermarking (fresh)-scent owner, as above. When the two males shared the same MHC type, which could not then be used for individual recognition, females discriminated a difference in their scents and spent longer sniffing the owner of the aged scent as if this was less familiar, as seen before (Figure 2B). This simple discrimination was expected in view of the large number of genetic and nongenetic factors that influence an individual's scent [1–3]. However, when males shared the same MHC type, females failed to show the usual strong attraction to the fresh-countermark owner; thus, there was no evidence of individual recognition (Figure 2B). Considered in isolation, this might suggest that MHC differences were essential to identify the individual males. However, when sib males differed in MHC type, females still failed to recognize

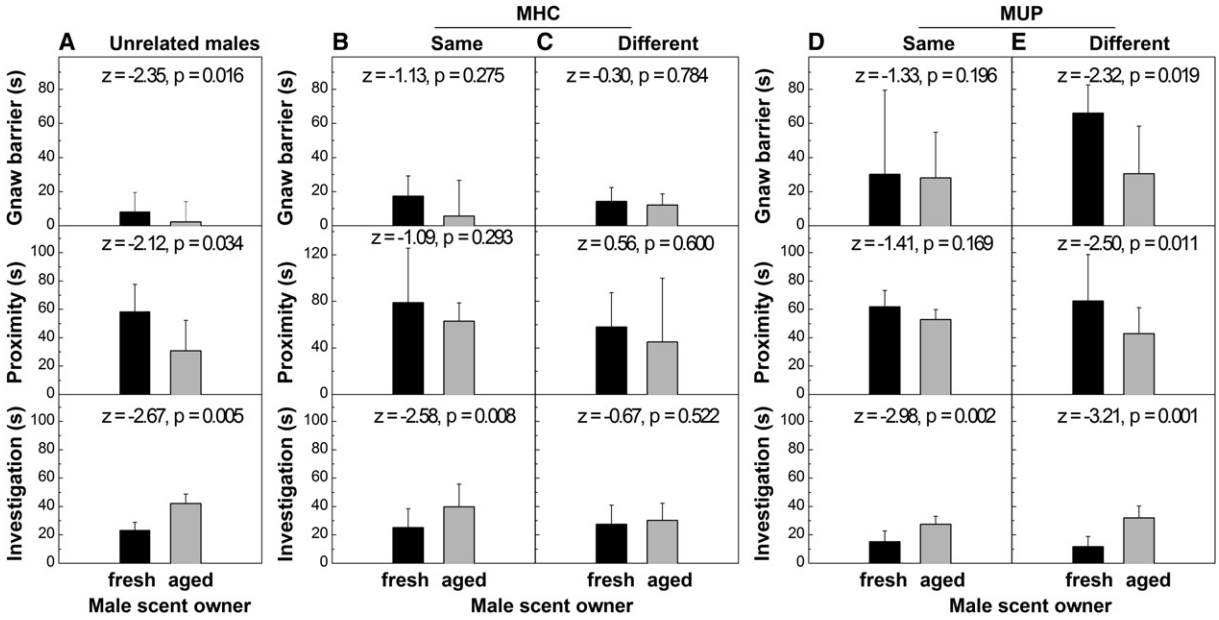

Figure 2. Recognition of Individual Males When One Male's Scent Countermarks the Other

After full contact with scent marks from two males genetically unrelated to each other (A), females were reliably more attracted to the owner of fresh scent marks (solid bars) over the owner of 24-hr-aged scent marks (gray bars). Attraction was evident from more prolonged gnawing of the barrier and time in proximity without interaction.

(B–E) Females were given a choice between two male scent owners that either had (B) identical MHC or (C) different MHC types or had (D) identical MUP or (E) different MUP types. Random variation in genetic background was insufficient to allow individual recognition, because females failed to show greater attraction to the fresh-scent owner when the two males shared identical MHC (B) or MUPs (D). Females also failed to identify the fresh-scent owner when the males had different MHC types (C), but correctly identified the males with different MUP types (E). Across all tests (with the exception of MHC different [C]; see main text), females investigated the donor of aged urine for longer, indicating that as expected, females detected differences between the male scents regardless of individual recognition. Data are median durations + 75% range, compared via Wilcoxon matched-pairs tests with exact probabilities.

the individual males (no difference in gnawing or proximity, Figure 2C). Thus, MHC was not used for individual recognition. Further, despite differing in MHC type and genetic background, both male scent owners were investigated equally (Figure 2C, discussed further below).

Individual Recognition Using MUP Type

The same tests were conducted with sib males of the same or different MUP type on variable genetic backgrounds. These showed that a difference in MUP type was essential for individual recognition. When males differed in MUP type, females recognized and were attracted to the countermarking male, spending more time trying to gain access through the barrier and more time in proximity to this male (Figure 2E). When sib males shared the same MUP type, there was no functional recognition: Females were not significantly more attracted to the owner of the fresh countermarks (Figure 2D; not significant even when gnawing of the barrier and time in close proximity were combined: $z = -1.37$, $p = 0.18$). Females predictably discriminated differences in the scents of these genetically diverse males regardless of any difference in MUP type, sniffing the owner of the aged scent more (Figures 2D and 2E), as in control tests with unrelated males. However, the information gained through sniffing only led to functional recognition of the countermarking male when males differed in MUP type. Interestingly, when males had different MHC types, and thus can be presumed to have readily discriminable differences in their scent profiles [2, 3, 6, 26–28], differential investigation of the two scent owners disappeared (Figure 2C). This might be because an obvious difference in MHC-associated scent profiles but not in the MUP signals used to recognize individual owners stimulated close inspection of both fresh and aged scents, such that both scents were then perceived as of equal familiarity or interest when females met the two scent owners.

If MUP, not MHC, provides the main genetic basis for individual recognition in mice, we predicted that females would recognize individual males in tests that manipulated MHC similarity when the two males differed in MUP type but not when they were the same (MUP type was originally ignored when assigning males to MHC same or different tests). To confirm this, we reanalyzed data from MHC tests (same and different) according to whether stimulus males shared the same or different MUP type ($n = 32$ tests). Combining both MUP and MHC datasets to maximize statistical power to detect individual recognition, there was still no evidence that females could recognize the fresh-scent owner when males shared the same MUP type (median duration of gnawing at barriers to fresh-countermark owner = 25.3 s, to aged-scent owner = 21.4 s; $z = -1.35$, $n = 31$, $p = 0.18$) but were strongly attracted to the countermarking male when males differed in MUP type (gnawing at barriers to fresh-countermark owner = 22.7 s, to aged-scent owner = 12.3 s; $z = -3.01$, $n = 40$, $p = 0.003$). We saw no evidence of stronger recognition when males

differed in MHC as well as MUP type. Females clearly discriminated differences between scents that were unrelated to MUP type in these genetically variable wild-derived males. This stimulated greater sniffing of the aged-scent owner as if this scent were more novel, whether the males' MUP patterns were different (median duration of sniffing at aged-scent owner = 32.1 s, at countermark owner = 17.9 s; $z = -4.01$, $p < 0.0001$) or the same (aged-scent owner = 30.6 s, countermark owner = 17.5 s; $z = -3.25$, $p = 0.001$). Nonetheless, this resulted in recognition of the individual males only when they differed in MUP type.

Discussion

Our results demonstrate that mice use polymorphic patterns of MUPs to recognize individuals, regardless of other genetic differences that influence an individual's scent, including MHC. Further, recognition requires direct contact with these urinary protein signals. Previous assumptions that any genetically determined differences in individual odor type must contribute to individual recognition (e.g., [1, 3, 26, 29]) are based only on the ability of animals to discriminate differences between scents, such as the increased investigation of novel scents in habituation-dishabituation tests. Like many other animals, mice sniff closely at the source of any unfamiliar airborne (volatile) scent to gain further information, but this response is nonspecific. The same response is induced whether scent is from animals of unfamiliar genetic identity or from familiar animals whose scent profiles have been altered, for example as a result of changed physiological status, diet, or bacterial flora [2, 14, 21]. This close-contact sniffing implies that animals do not acquire sufficient information from the airborne scent profile alone to interpret a novel scent signal. In most nonhuman tetrapods, close-contact investigation allows animals to detect involatile as well as volatile scent components through the vomeronasal system, which responds specifically to conspecific and heterospecific chemical stimuli with important intrinsic meaning for the species [6, 18, 30]. It is only after acquiring scent information through contact that mice are able to identify individual scent owners. This is not driven by any discriminable difference in individual scents but by MUP type as a specific signal of individual identity. This is consistent with earlier findings that male mice recognize their own scent marks or those from other males entirely on the basis of contact with MUP pattern, with recognition disrupted by addition of a recombinant MUP [13, 22, 31]. Importantly, our current study extends this to the genetic basis for recognition between equivalent individuals and demonstrates that the same recognition signal is used in these different social contexts. Why should animals focus on a specific signal to identify individual conspecifics regardless of other scent differences between them? This allows animals to use a fixed indicator of genetic identity that is easily recognized and distinguishable from variation caused by current state or environment. For example, the species-specific facial characteristics used for visual individual recognition by primates including humans [32] are robust to changes in facial expression that reflect emotional state or behavior rather than identity [33].

Polymorphic urinary MUPs have evolved exclusively for scent communication, with specialized characteristics to fulfill this role. Mice excrete a high concentration of 8–14 electrophoretically separable MUP isoforms in their urine. These MUPs provide each individual with a distinctive signature that is highly resistant to degradation in scent marks and can be distinguished regardless of the complex internal and external factors that influence volatile scent profiles ([9, 13]; A.L. Sherborne, M.D.T., S. Paterson, F.J., W.E.R.O., P. Stockley, R.J.B., and J.L.H., unpublished data; and present study). The central cavity of MUPs binds low-molecular-weight volatile pheromones, integrating identity and status information [8, 9]. Some MUP polymorphic variants differ in their affinity for ligands bound in the central calyx [8, 34, 35], although most heterogeneity resides on the surface of the protein rather than at the ligand-binding site [36]. Further, the proteins themselves appear to be detected given that both MUPs stripped of ligands and synthetic MUP peptides trigger ovulation through the vomeronasal system [10]. Thus, the individual signature could consist of MUP-ligand complexes or MUPs alone. Once individuals are identified through this involatile fixed identity signature, a learned association with airborne volatiles detected simultaneously through the main olfactory system would allow familiar scents to be recognized from airborne volatiles without the need to recontact the fixed identity signature [14]. Given that many nongenetic and genetic factors (including MHC) contribute to an animal's airborne volatile profile, these are likely to play an important role in drawing attention to unfamiliar scents or allowing animals to quickly recognize familiar scents that are associated with known MUP identity signatures. However, such recognition of familiar airborne scents represents the recognition of "familiar" versus "novel," not individual recognition per se. Individual recognition will still depend on prior contact with the involatile MUP identity signature, requiring reinvestigation whenever the animal's more labile volatile profile changes or the association is forgotten.

The ability to *discriminate differences* between MHC-associated scents derived from laboratory mice is well established [2, 3, 6, 26, 37], but we found no evidence that MHC variation among wild mice contributes to the *recognition of individuals*. Other studies have not examined responses to normal variation in MHC types derived from wild mice, and very few have used a functional test of individual recognition. Pregnancy block in laboratory mice (the Bruce effect) in response to males with different MHC signals from the familiar sire has been widely interpreted as signifying individual recognition [6], although the perceptual meaning of differences that induce pregnancy block is still unclear. After females have learned the scent of a male during mating, exposure to scent from a male of an unfamiliar laboratory strain causes abortion through failure of embryo implantation [4, 38]. Foreign peptides that cannot bind to the familiar sire's MHC proteins produce the same effect when added to urine from genetically identical males, suggesting that females may recognize urinary peptides corresponding to the owner's MHC type [27]. However, pregnancy is not blocked by MUP differences between strains [39] even though this signal clearly underlies

individual recognition in more general social contexts among wild mice. One possibility is that mice use a different system for recognizing individual males in the context of pregnancy block. However, an extensive study of wild mice failed to demonstrate pregnancy block simply in response to unfamiliar individuals; instead, this depended on the t complex genotype of the unfamiliar male [40]. This structurally variant segment of chromosome 17 includes the MHC [41], and t haplotype carriers have unique MHC alleles with unusually low polymorphism [41, 42]. The response of wild mice is thus consistent with sensitivity to variation in this area of the genome, but it is not yet known how this influences urinary peptides that induce pregnancy block. It is essential to define the repertoire of peptides in the urine of individual wild mice and their origins to further understand the functional significance of pregnancy block and the signals that induce this response.

To our knowledge, this is the first study to establish the genetic basis of signals used to recognize individual identity in any vertebrate by using appropriate functional tests and natural variation between animals. This reveals that mice depend on a specialized set of highly polymorphic signaling proteins (MUPs) to recognize individuals. Recently, we have also shown that the same genetic identity signal is used to avoid inbreeding with very close relatives (A.L. Sherborne, M.D.T., S. Paterson, F.J., W.E.R.O., P. Stockley, R.J.B., and J.L.H., unpublished data), contrary to the suggestion that MHC-associated odors provide a vertebrate-wide mechanism for recognition of individuals and kin. However, other factors that influence scents (including MHC) may contribute to the recognition of familiar animals (rather than individuals), which might be important in other social contexts such as mother-offspring recognition. The extreme polymorphism in MUP patterns expressed by house mice may have evolved as a result of a need for reliable individual and kin recognition as an adaptation for living in dense aggregations [43]. In common with MHC genes used for self/nonself recognition at the molecular level, MUP genes are inherited as a tightly linked cluster or haplotype [14]. Inbreeding avoidance, promiscuity, and offspring dispersal are all likely to contribute to maintaining the substantial individual variation found in MUP patterns in natural mouse populations. At present, little is known about how widespread MUP-like orthologs are in animal scents, although these appear to be common across murid rodents [12]. Further research is required to understand the evolution and maintenance of this MUP polymorphism, and the extent to which MUP-like orthologs have evolved to allow reliable genetic identity recognition through scent or through alternative signaling modalities in other species.

Experimental Procedures

Subjects and Stimulus Animals

Female subjects were selected from a pool of wild house mice (*Mus musculus domesticus*) caught from populations in the northwest of England several months prior to the experiment (n = 68) or were first-generation captive bred (n = 27). Male stimulus animals were first- to third-generation captive-bred mice (n = 143) or captured from the wild (n = 17). Females were tested with males that were unrelated and unfamiliar. Females were housed in small groups of two to four (cages 40 × 23.5 × 12.5 cm). Males were weaned into single-sex groups at 4 weeks of age and then individually housed from 8–10 weeks (cages 43 × 11.5 × 12 cm) because wild-derived adult males frequently become highly aggressive and intolerant of cage mates. All mice were provided with water and food ad libitum (TRM9607, Harlan Teklad) and were maintained on a reverse 12 hr:12 hr light cycle. Mice were 6–18 months old at the time of testing.

To bring females into a sexually receptive state [44] and ensure that all subjects and stimulus animals had prior experience of odors from the opposite sex, we primed all mice with soiled bedding from the opposite sex in their home cages 3 days prior to their use in a test. Soiled bedding was not from individuals that would be encountered during tests. Female estrus state was confirmed by vaginal smear immediately after testing for a subsample of females used in this and a related study in our laboratory (96% of 90 females tested had > 50% cornified cells and were in estrus or proestrus). Tests were carried out during the dark phase under dim red light. If females were used in more than one test condition, these were conducted at least 4 weeks apart and involved different stimulus males. Urine was obtained by holding a stimulus male by the scruff of the neck over a clean 1.5 ml Eppendorf tube. Urine was collected up to 2 weeks prior to testing and stored at −18°C until use.

Recognition with or without Scent Contact

We tested recognition of the owner of a familiar scent by presenting females with urine streaks from an unrelated male in the identity-learning phase of the test; they were then given a choice between the scent owner and an equivalent unfamiliar male to test whether they recognized the familiar-scent owner in the recognition phase of the test (males presented behind mesh barriers [minimum 4 × 3 mm mesh size] to allow olfactory, visual, and some physical contact while preventing further contact with scent marks). Male stimulus animals were used as the familiar-scent donor for one female and as the novel individual for another female, to control for any variation in individual male quality. To test whether recognition depended on contact with a male's scent marks, we either allowed females to contact the urine streaks during investigation in the identity-learning phase (n = 12) or prevented contact by using a double layer of mesh so that only volatile information from the scent was available in the identity-learning phase (n = 12). Male urine (20 μl) was deposited in two 10 μl streaks on glass microfiber filters (Whatman circles, grade GF/C, 70 mm) placed in the center of a clean cage (29 × 11.5 × 12 cm), and a female was introduced for 15 min. The female was then transferred to a clean test arena (40 × 23.5 × 12.5 cm high with clear perforated acrylic lid) in which the scent owner and an unfamiliar male were held in two mesh-capped Perspex tubes (190 × 40 mm diameter) inserted into opposite walls of the arena (the mesh caps protruded approximately 2 cm into the arena). Each test (15 min) was video recorded remotely under dim red light. As an unambiguous measure of attraction to each male, we recorded the time that females spent gnawing and pulling at the mesh barriers actively trying to get to each male over the 15 min test. This is a measure of attraction to the male rather than aggression because females in this species rarely show overt aggression toward males unless pregnant or lactating. We also recorded the time spent investigating each male through the barrier to gain further information (sniffing at or through each barrier) and time spent in proximity without interacting with the male or barrier (within a 24 × 10 mm rectangle in front of the barrier, marked on Benchkote lining the arena floor).

Individual Recognition

Females were presented with urine streaks from two males deposited as if one male had countermarked the other in the identity-learning phase in order to test whether they could recognize individual males from their scents when both scents were familiar. They were then given a choice between the two male scent owners in the test arena described above in the recognition phase of the test. Urine (20 μl) from the first male was deposited in two 10 μl streaks on a glass microfiber filter, and the scent marks were left in the open at room temperature (21°C) for 24 hr. The same amount of urine from a second male (the countermarking male) was then deposited as fresh scent in four 5 μl streaks, 5 mm on either side of the 24-hr-aged scent marks. Females were presented with the scent marks in a clean cage for 15 min with full contact before transfer to a test

arena containing both scent owners behind mesh barriers for a 15 min choice test as described above.

We first confirmed that females could recognize which of two scent-mark owners had deposited the fresh countermarks when males were unrelated and thus genetically distinct at MHC, MUP, and genetic background (n = 12). To assess whether MHC or MUP were necessary for individual recognition of scent owners, we tested females with pairs of males that either had the same or different MHC type (with random differences in MUP and genetic background) or had the same or different MUP type (with random differences in MHC and genetic background). Because both MHC and MUPs are so genetically heterogeneous among wild mice, only some closely related males share the same MHC or MUP types. In these tests, stimulus males were pairs of full sibs that were unrelated and unfamiliar to the test female. Litters containing three or more males were used in which one male (assigned as the countermarking male) could be paired with a sib of the same type and with a sib of a different type in separate tests using different females. The sample size was increased to n = 20 for the four tests using sib males in case discrimination between two sibs was more difficult than between two unrelated males.

MHC and MUP Typing

MHC and MUP type were established by genotyping parents and male offspring to identify haplotypes using microsatellite markers (six markers across the MHC region on chromosome 17 and eight markers surrounding the MUP region on chromosome 4). DNA was extracted from a 1–5 mm tail snip with mouse-tail extraction kits (Tepnel life sciences). Twelve markers were originally selected for the MHC region (chromosome 17) with the ENSEMBL mouse genome database. Three markers were deemed outside the MHC region (confirmed by crossover events in test samples, F.J. and W.E.R.O., unpublished data) and were removed from the analysis. Three other markers were excluded from analysis because of amplification problems. Eleven markers were identified in the putative MUP 4 locus (chromosome 4). Three of the markers failed to amplify or were found to be monomorphic in wild mice and were removed from the data set. The 14 chosen loci are listed in Table S1. The primer sets were designed so that markers would fall into one of three size groups with nonoverlapping allele lengths with the forward primer in each group 5′-end-labeled with a fluorescent phosphoramidite (6-FAM, HEX, or NED). The loci were organized into four multiplex loading groups, containing mixed loci from both the MHC and MUP regions.

PCR amplification reactions were performed in a final 10 μl volume containing 10 ng of DNA, 0.1 μM of each primer, 0.2 mM of each dNTP, 0.05 units of Taq DNA polymerase (Hotstar Taq, QIAGEN), and 2.5 mM MgCl2 in the supplied reaction buffer. PCR amplification was performed on a thermal cycler (GRI) with a touchdown PCR program at one cycle of 95°C for 15 min to activate the Taq polymerase and then 94°C for 20 s, 30 s at 66°C, −0.5°C per cycle for 14 cycles, and then 60°C for 20 cycles and 72°C for 30 s, followed by 72°C for 10 min. For each individual sample, PCR products from each multiplex group were diluted 1 μl for 6-FAM-labeled products and 2 μl for the other labeled products into 200 μl ddH20. The multiplexed mixture (1.2 μl) was combined with 5 μl deionized formamide and 0.1 μl internal lane standard (ROX400, Applied Biosystems). Samples were electrophoresed on a 3100 Genetic Analyzer capillary electrophoresis system with a 22 cm array (Applied Biosystems). The fluorescence emission for each dye was collected and analyzed for size variation with the Applied Biosystems GENESCAN (version 3.7) and GENOTYPER (version 3.7) DNA-fragment analysis software.

We confirmed that same or different MUP genotypes corresponded with same or different phenotypes by isoelectric focusing of urine samples from each mouse to compare the patterns of MUPs expressed [13].

Data Analysis

The durations of gnawing or pulling at the mesh barriers to get to each male, investigation of the barrier or male, and time spent in proximity without interaction were transcribed from video tapes with an event recorder program (written by R.J.B.). The observer was blind to the identities and genotypes of the stimulus males. The difference in response to the two stimulus males was assessed with nonparametric Wilcoxon matched-pairs tests (two tailed) because behavioral data were not normally distributed.

Supplemental Data

One table is available at http://www.current-biology.com/cgi/content/full/17/20/1771/DC1/.

Acknowledgments

We thank Dr. Richard Humphries, Linda Burgess, John Waters, and Sue Jopson for practical help; Dr. Jonathan Mudge for MUP locus information; and Drs. Paula Stockley and Steve Ramm for comments on an early draft of the manuscript. The work was supported by the Biotechnology and Biological Science Research Council (grant S19816 and studentship 02/A1/S/08188).

Received: August 8, 2007
Revised: August 31, 2007
Accepted: September 1, 2007
Published online: October 18, 2007

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