Please cite this article in press as: Thom et al., The Direct Assessment of Genetic Heterozygosity through Scent in the Mouse, Current Biology (2008), doi:10.1016/j.cub.2008.03.056

Current Biology *18*, 1–5, April 22, 2008 ©2008 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2008.03.056

Report

# The Direct Assessment of Genetic Heterozygosity through Scent in the Mouse

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## Summary

**The role of individual genetic heterozygosity in mate choice is the subject of much current debate [1–6]. Several recent studies have reported female preference for more heterozygous males [7–9], but the mechanisms underlying heterozygote preference remain largely unknown. Females could favor males that are more successful in intrasexual competition [10–15], but they could also assess male heterozygosity directly at specific polymorphic genetic markers [9, 16–18]. Here, we use a breeding program to remove the intrinsic correlation between genome-wide heterozygosity and two highly polymorphic gene clusters that could allow direct assessment of heterozygosity through scent in mice: the major histocompatibility complex (MHC) [19–21] and the major urinary proteins (MUPs) [22–24]. When other sources of variation are controlled and intrasexual competition is minimized, female mice prefer to associate with MUP heterozygous over MUP homozygous males. MHC heterozygosity does not influence preference, and neither does heterozygosity across the rest of the genome when intrasexual competition between males is restricted. Female mice thus assess male heterozygosity directly through multiple MUP isoforms expressed in scent signals, independently of the effects of genome-wide heterozygosity on male competitiveness. This is the first evidence that animals may use signals of genetic heterozygosity that have no direct association with individual vigour.**

## Results

To disentangle the link between heterozygosity at major urinary protein (MUP), major histocompatibility complex (MHC), and across the genome, we utilized the genetic control provided by inbred laboratory mouse strains to generate stimulus animals, while using wild-derived female house mice (*Mus musculus domesticus*) as subjects to ensure natural sociosexual responses to the manipulated males [25]. Two inbred strains from separate genetic lineages (C57BL/6 and BALB/c, which differ in MUP and MHC type, as well as many other genetic loci) were crossed to produce male pairs of heterozygous versus homozygous F2 segregants at either MUP or MHC (the other held constant) with equivalent levels of background heterozygosity. As a control, we also paired age-matched F1 and F2 males that differed in heterozygosity across the genome except at MUP and MHC. To assess female preference, we placed two males in a pair of linked enclosures (each 1.2 m × 1.2 m) connected by a narrow tunnel that the female could pass through while the larger males were each confined to their own territory. Thus, females were able to choose between adjacent territorial males differing in heterozygosity in the absence of intrasexual competition that could influence their relative social status. Before assessing female response to males, we first assessed any preference between the two scent-marked territories when males were removed for 7 hr. Preference when males were returned to their territories was then assessed by monitoring of the locations of both the female and the males continuously over 78 hr (see the Supplemental Experimental Procedures available online for further details). This encompassed a full female oestrus cycle: Most females were expected to enter oestrus around 72 hr after first being introduced to the enclosures [23].

### Female Preference

In the absence of the male territory owners, females showed no bias in time spent in the scent-marked territories or nest sites of heterozygous males, whether males differed in heterozygosity at MUP, MHC, or across the rest of the genome (Table S3). However, when males were present in their territories, females spent more time overall coinhabiting the nest of the MUP heterozygous male than that of the MUP homozygous male ($F_{1,13}$ = 5.95, p = 0.03, Figure 1). This bias was evident in each separate phase of the light cycle after the first 24 hr of interaction (Figure 2), and a repeated-measures analysis confirmed that females showed a consistent preference for coinhabiting the nest of the MUP heterozygous male during both the dark ($F_{1,13}$ = 9.89, p = 0.008) and the light ($F_{1,13}$ = 5.36, p = 0.038) phases of the circadian cycle. This preferred association with MUP heterozygous males appears to be due to females seeking or accepting close contact with the males within their nest sites. There was no bias in the total time spent within the MUP heterozygous versus homozygous males' territory overall (Table S3) or in the time that females spent alone within each nest when the male was elsewhere in the territory ($F_{1,13}$ = 0.92, p = 0.36). Neither was the bias due to any difference in the time spent by the males themselves alone within nest sites (MUP heterozygous versus homozygous male: light phase, 56 ± 5% versus 40 ± 7%; dark phase, 11 ± 2% versus 12 ± 2%; $F_{1,13}$ = 0.63, p = 0.44).

In contrast, MHC heterozygosity did not significantly influence time spent in close association with males or total time spent within each male's territory (Table S3). Further, when MUP and MHC heterozygosity were simultaneously held constant, heterozygosity across the rest of genome did not influence female-preferred association between the two male territory owners (Table S3).

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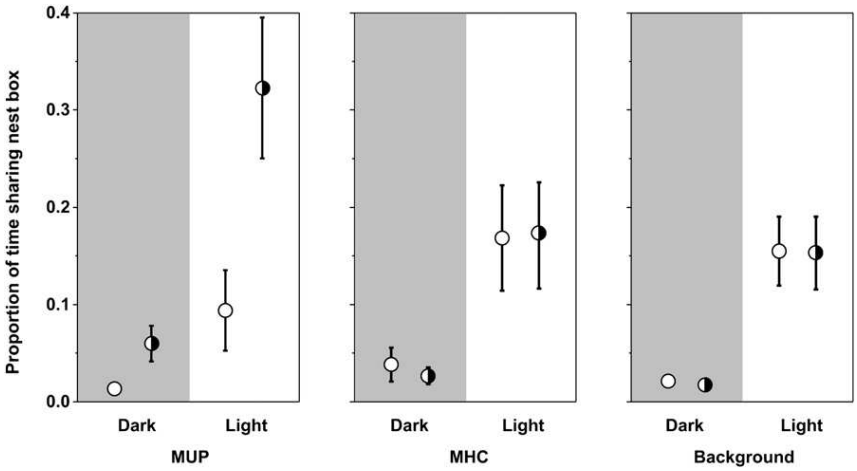


Figure 1. Preference for Nesting with Homozygous or Heterozygous Males

Proportion of total time that females spent nesting with males that were either homozygous (open circles) or heterozygous (half-filled circles) at MUP, MHC, or across the rest of the genome (separate panels), summed separately across the dark (shaded background) and light (unshaded background) phases (mean ± standard error [SE]). Animals spent more time nesting—both alone and together—during the light phase across all tests, and hence the lighting-phase term is significant in all models of time spent in nest boxes. Male heterozygosity across the genome and at MHC type had no effect on nest-box use by females. In contrast, females nested for significantly longer with MUP heterozygous males than with MUP homozygotes ($p = 0.03$); this behavior was not significantly influenced by lighting phase (interaction term: $p = 0.12$; see text for details).

## Male Aggression

We deliberately minimized intrasexual competition among males by ensuring that each male occupied an equivalent territory regardless of individual aggressiveness or competitive ability, although males were allowed to interact in a short series of encounters prior to female introduction to encourage normal development of male competitive behavior and scent production (see the Supplemental Experimental Procedures). Because the *Mup* gene cluster encodes only special communication proteins in a male's scent, it is unlikely that variation at this region would influence male aggressiveness. Nonetheless, to check that this did not explain female preference for MUP heterozygous males, we classified males according to whether the heterozygous or homozygous male was the more aggressive within each pair in encounters prior to female introduction. Overall, we confirmed that there were no significant effects of MUP or MHC heterozygosity on the aggressiveness of males when they were allowed to interact (number of aggressive behaviors initiated: Wilcoxon test, MUP: $z = -0.70$, $p = 0.48$; MHC: $z = -0.44$, $p = 0.66$) or on the frequency with which they were first to invade their neighbor's territory (MUP: $z = -0.74$, $p = 0.46$; MHC: $z = -1.11$, $p = 0.27$). With a binary variable describing male aggression included in the model, the females' consistent preference for associating with MUP heterozygous males remained (main effect of heterozygosity: $F_{1,12} = 6.42$, $p = 0.026$), but there was no interaction with male MUP heterozygosity (interaction term: $F_{1,12} = 2.02$; $p = 0.18$), indicating that preference for MUP heterozygotes was not influenced by relative aggression of the male.

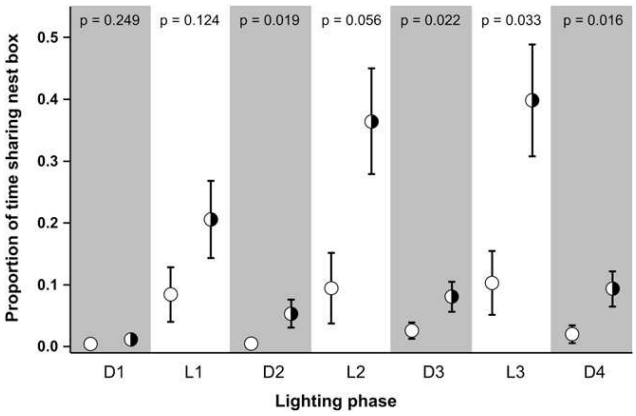


Figure 2. Time Course of Preferred Nesting with MUP Heterozygous Males

Proportion of total time that females nested with the MUP-homozygous (open circles) or MUP-heterozygous (half-filled circles) male during the course of the trial, with the four dark (shaded background) and three light (unshaded background) phases shown separately (mean ± SE). Females spent significantly longer nesting with the MUP heterozygous male in the majority of light and dark phases (post-hoc Wilcoxon paired tests; p values shown in the figure). Inclusion of all light periods in repeated-measures tests reveals a reliable effect of male genotype on female preference during the three light phases ($p = 0.038$), and the four dark (active) phases ($p = 0.008$; see text for details). Most females were likely to be in oestrus during D3.

Heterozygosity across the genome, on the other hand, is known to have a major effect on the ability of male mice to defend territories [12]. In separate tests where 11 pairs of males differing in heterozygosity across the genome (MUP and MHC held constant) were allowed to interact frequently to establish a dominance relationship within cages (see the Supplemental Experimental Procedures), the relatively heterozygous (F1) male became dominant over the relatively homozygous (F2) male in every case (binomial test: $p < 0.001$). We ensured that differences in intrasexual competition were minimized for the purposes of the female preference test by allowing each male to inhabit an equivalent territory. However, males that were more heterozygous across the genome (excluding MUP and MHC) were three times more likely to intrude first into the neighboring territory when allowed to interact before females were introduced (the more heterozygous male was the most frequent first intruder in 12 out of 16 experimental pairs and entered first on a mean 3.4 out of 5 opportunities compared with a mean 1.1 out of 5 opportunities for the homozygous male: Wilcoxon test $z = -2.91$, $p = 0.004$). This did not lead to any consistent difference in their aggressiveness during these initial interactions (the heterozygous male showed more aggression in 9 out of 16 pairs, $z = -0.82$, $p = 0.41$) and, even when relative aggressiveness was taken into account, females failed to associate more with males with greater genome-wide heterozygosity (excluding MUP and MHC) when both males inhabited similar territories (main effect of heterozygosity: $F_{1,13} = 0.043$, $p = 0.84$; interaction between heterozygosity and aggression: $F_{1,13} = 0.012$, $p = 0.92$).

## Mechanism of Assessment

To examine further the mechanism that females use to discriminate between MUP heterozygous versus homozygous

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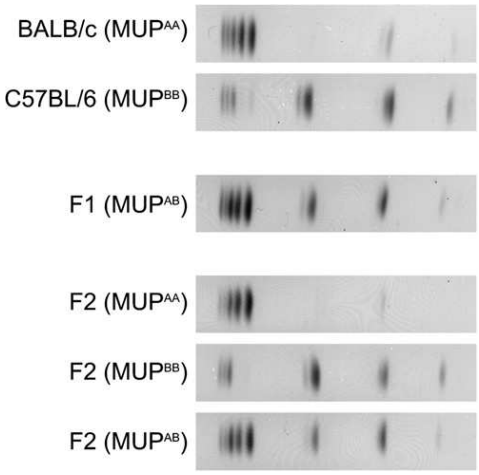


Figure 3. MUP Complexity According to Male MUP Genotype

Isoelectric focusing gel showing individual MUP isoforms separated by charge. Whole urine samples were diluted to a standard protein concentration of 1 mg/ml before the gel was loaded. From bottom to top, the lanes show the MUP patterns for the parental (F0) homozygous inbred strains, the heterozygous F1s, and the homozygous and heterozygous F2s. All samples are from adult male mice.

males, we analyzed the MUPs expressed in the urine of males with different MUP genotypes. We first confirmed that females preferred to associate with MUP heterozygous males over both types of homozygote: Although heterozygote MUP$^{AB}$ preference tended to be stronger (Figure S1) when matched against homozygous MUP$^{AA}$ compared to MUP$^{BB}$ males, this effect was not significant (interaction term: $F_{1,24} = 2.09$, $p = 0.16$). Overall, there was no difference in the total concentration of urinary protein expressed by MUP heterozygous males compared to the two homozygous phenotypes (for a subset of 11 male pairs, urinary protein to creatinine ratio to correct for urine dilution [26]: MUP$^{AA}$ 18.1 ± 2.5, MUP$^{BB}$ 19.6 ± 0.8, MUP$^{AB}$ 21.6 ± 2.0, Kruskal-Wallis $\chi^2 = 0.26$, $p = 0.88$). Isoelectric focusing, which separates MUP isoforms by charge, confirmed that heterozygotes express all protein bands found in both homozygotes (Figure 3), consistent with codominant expression. Intact mass profiling indicates that the amount of each MUP isoform expressed by heterozygotes is intermediate between the two homozygous phenotypes (M.D.T., J.L.H., and R.J.B., unpublished data), and although MUP$^{AB}$ heterozygotes express no more male-specific MUPs than do MUP homozygous males, homozygous MUP$^{AA}$ males express no detectable male-specific 18893Da MUP (J.L.H. and R.J.B., unpublished data) [27]. This male-specific MUP is expressed by most wild male mice and is responsible for binding most of the male pheromone 2-*sec*-butyl 4,5 dihydrothiazole in mouse urine [28]. The absence of this male-specific protein might have contributed to the stronger preference for MUP$^{AB}$ heterozygotes when paired with homozygous MUP$^{AA}$ males (Figure S1). However, MUP$^{AB}$ heterozygotes expressed no more of this MUP than did MUP$^{BB}$ homozygotes, so the only major difference in phenotype between MUP heterozygous and homozygous males is in the number of different MUP isoforms expressed. Although each of the homozygous phenotypes express four different main urinary MUP isoforms (J.L.H. and R.J.B., unpublished data) [27], at least seven different isoforms are expressed by heterozygotes, suggesting that the most likely mechanism underlying the direct assessment of male heterozygosity is through detection of the greater number of MUP isoforms in heterozygotes.

## Discussion

Our results demonstrate that female mice use a specific genetic marker, the MUPs, to identify and preferentially associate with heterozygous males. The primary function of MUPs is in signaling social information through scent [22, 24, 29, 30], including individual genetic identity [23]. Heterozygosity at this region has no known or expected effects on the health or vigor of the signal owner. Thus, our study presents the first evidence of a mechanism used by animals to assess genetic heterozygosity directly where the genetic marker used is unlikely to directly influence individual condition. This discovery has significant implications for the debate concerning the role of genetic diversity in mate choice [3] because it demonstrates that assessment of male heterozygosity per se may be important to females, regardless of condition-dependent traits that may be influenced by heterozygosity.

The high polymorphism of each of the putative signaling systems tested (MHC and MUP) is sufficient to ensure that diversity of each signal reflects overall genetic diversity. Odors associated with MHC heterozygosity influence female association with males among sticklebacks [17, 18], and there is evidence of female preference for the odors of MHC heterozygous males in humans [31]. MHC odors have also been implicated in mate choice and social association in the mouse [21, 32, 33], although not in the context tested here. Genetic heterozygosity at MHC is thought to be associated with increased health and vigor [20, 34], so a preference for MHC heterozygotes could be based on diversity at the MHC itself or a response to the better overall condition of more heterozygous males. There is an additional difficulty in studying female preference for MHC heterozygotes: MHC diversity will normally correlate with genome-wide heterozygosity in natural populations (a supposition that has mixed empirical support [3], pp. 207–208). This association is not controlled in natural population studies such as those on sticklebacks and humans, so the possibility that females use other markers in linkage disequilibrium with MHC has not been eliminated. Here, after experimentally disentangling MHC and genome-wide diversity, we found no evidence that MHC heterozygosity alone influences female association with males in mice, although mice have the potential to assess MHC diversity directly [19] as in sticklebacks [17].

Instead, we found that heterozygosity at the MUP region plays a significant role in female association behavior. It is possible that differential investment in MUPs could influence male condition through loss of valuable protein from the body, but both protein concentration and body mass were equal between MUP heterozygous and homozygous males in our study. Thus, it is extremely unlikely that females detect diversity at this region through effects on male condition, further confirmed by similarity in levels of aggression between the males. MUPs are primary gene products that provide a direct marker of genetic identity in mouse scents [35, 36], and these specialized signaling proteins are detected through a class of receptors in the vomeronasal organ [22]. Mice are highly sensitive to differences in the fixed patterns of multiple MUP isoforms expressed by each individual [23, 24] and also avoid inbreeding with close relatives that share the same MUP type as themselves [37]. Heterozygosity assessment, in contrast, most likely involves recognition of the greater diversity of MUP isoforms expressed by heterozygous animals.

Our discovery that female mice use the MUP region as a mechanism for assessing heterozygosity takes us a step closer to identifying the potential benefits of female preference for more heterozygous males [1, 3, 9]. We did not measure mate choice directly—under captive conditions, wild mice do not breed as easily as laboratory strains that have been selected for this over many generations and even mate with very close relatives. This emphasizes the importance of using wild-derived subjects with normal behavior [25] and natural levels of genetic variability to understand social preferences and mate choice decisions [23, 37]. If the observed preference does extend to mate choice, this could have genetic benefits because of the normal correlation between MUP and genome-wide heterozygosity. Although heterozygosity is not directly heritable, parent-offspring heterozygosity correlations can occur in wild populations [3, 9, 38], and females choosing heterozygous males may experience greater than average fitness in a fluctuating environment [39]. There may also be benefits to increasing the diversity of offspring specifically at the *Mup* gene cluster because individual variation in MUP patterns is essential for individual recognition [23]. However, preferred association with MUP heterozygous males may reflect a preference for the nests or territories of these individuals without a sexual preference for such males. The substantial direct benefits potentially offered by more heterozygous males, as illustrated by the significantly greater survival and territorial ability of outbred mice in seminatural enclosures [12], may have a considerable impact on female reproductive success irrespective of whether the heterozygous male is also the sire. A direct signal of genetic heterozygosity, in addition to any signals that indicate the current competitive success of a male such as territorial countermarking [40], may provide information about the male's potential vigor and success in future competition. Alternatively, preference for heterozygosity may not be specific to the opposite sex, and females may avoid nesting in sites where signals of homozygosity indicate inbreeding within the local population. In order to distinguish between these possibilities, we are currently undertaking longer term experiments to test whether the association preference for MUP heterozygous males leads to a mating preference, supporting the hypothesis that sire heterozygosity has genetic benefits for offspring.

The MUP signaling system is central to individual recognition and to the maintenance of outbreeding through kin recognition in the house mouse [23, 36, 37]. We now have evidence that females are sensitive not only to the degree of MUP matching with potential mates but also to genetic heterozygosity at this region within individual males. The central role of MUPs in individual recognition, kin avoidance, and heterozygosity assessment make this an ideal system for addressing the function of genetic signals in social and mate choice in vertebrates.

### Supplemental Data

Experimental Procedures and one figure are available at http://www.current-biology.com/cgi/content/full/18/8/■■■/DC1/.

### Acknowledgments

We thank Richard Humphries, Sarah Cheetham, Linda Burgess, Felicity Fair, Sue Jopson, and John Waters for practical help. Thanks to Mike Francis for supplying the automated animal tracking system and software. This work was supported by the Biotechnology and Biological Science Research Council (S19816).

Received: February 25, 2008
Revised: March 17, 2008
Accepted: March 27, 2008
Published online: April 17, 2008

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# Supplemental Data



# The Direct Assessment of Genetic Heterozygosity through Scent in the Mouse

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## Supplemental Experimental Procedures

The animal procedures used in this study were approved by the United Kingdom Home Office and the Animal Welfare Committee of the University of Liverpool, United Kingdom.

### Subject Females

We used wild-derived house mouse (*Mus musculus domesticus*) females from our laboratory-maintained breeding stock as subjects. These were first- to fifth-generation captive bred animals derived from wild-caught animals from various locations around the United Kingdom. Females were aged between 33 and 74 weeks (mean = 47 ± 1.8) at the time of testing; these animals had sometimes been used in previous behavioral experiments, but none had reproductive experience and none had previously encountered the males with which they were paired in this experiment. Females were housed in single-sex family groups of two to five individuals until testing.

### Experimental Males

We produced experimental males by crossing two inbred laboratory strains to create stimulus males that varied in heterozygosity specifically at MHC, MUP, or genetic background. The homozygous MHC and MUP types of the two parent strains (BALB/c and C57BL/6 obtained from Harlan UK) have been well characterized, and there are multiple differences between the strains in MHC [S1] and MUP type (J.L.H. and R.J.B., unpublished data) [S2], as well as across the rest of the genome [S3, S4]. F1 crosses were thus heterozygous across the genome, including at the MUP and MHC regions of interest, whereas F2s segregated into nine combinations of MUP and MHC type (Table S1). We typed all F2s for MUP and MHC and selected male pairs for mate choice tests so that one male was heterozygous and one male homozygous at the region of interest (either MUP or MHC), with heterozygosity at the other region held constant (Table S2). To assess the importance of heterozygosity at unmeasured genetic background loci, we selected F2 males with heterozygous MUP and MHC types and paired these with F1 males, which were also MUP and MHC heterozygous. F1 males are on average twice as heterozygous as F2 males at all other loci, mimicking an inbred and an outbred male with the putative signaling systems controlled. Males were aged between 20 and 56 weeks at time of testing (mean 40 ± 1) and were age matched as closely as possible (mean 4.6 days, maximum 40 days age difference). We reused males a maximum of twice; in each instance of reuse, males were assigned to a new pair with a male that had also been used once before to control for experience.

### Experimental Enclosures

We tested female association behavior in linked pairs of relatively large (1.2 m × 1.2 m) indoor enclosures to simulate the natural situation where females would encounter males in their own territories. Each pair (n = 8 pairs) of enclosures was connected by a tunnel (325 mm × 50 mm diameter; hereafter the link tunnel), and each enclosure was provided with a nest box (L × W × H: 119 mm × 119 mm × 90 mm) containing nesting material (3 × hemp fiber Happi-Mats, IPS Product Supplies, UK). A second, blind-ended tunnel (den; 150 mm × 50 mm diameter) was fixed against one wall as a second refuge, and the bases of four small (335 mm × 160 mm × 116 mm) rat cages were placed on the floor of the enclosure to provide three-dimensional complexity to the territory. Food (Lab Diet 5002 Certified Rodent Diet) and water were provided ad libitum in a standard mouse cage lid (480 mm × 115 mm) placed on the enclosure floor.

We used an automated data-logging system to record both male and female location during each trial. Each animal was individually tagged with a radio frequency identification (RFID) tag beneath the skin at the nape of the neck. Automated RFID readers housed in clear Perspex boxes (110 mm × 126 mm × 115 mm; Francis Scientific Instruments [FSI], Cambridge United Kingdom) were placed over each end of the link tunnel, the den, and the entrance tunnel to the nest box of each enclosure. Each reader incorporated an infrared beam and associated detector, which in combination with the RFID detector allowed us to assign the direction of movement and hence the location of animals within a nest box, within a den, within the link tunnel or outside in one of the open enclosures. A computer running custom software (FSI) tracked the movements of animals within the enclosures: Each time an animal passed through a detector, the unique RFID code, time, date, and direction of travel were logged to a central computer. Because female mice typically range over several male territories during normal activity, the overall time spent within each territory may not reflect preference for interacting with the territory owners. To assess close association with each male, we thus measured the time that the female spent together with the male inside his nest box.

### Male Habituation and Aggression

Males were placed into their separate enclosures prior to the female choice test, to allow them to habituate to the apparatus, to develop normal territorial behavior, and to scent mark their territories. Initially, the link tunnel was obstructed at both ends with a wire-mesh cap, allowing males only visual and olfactory contact with each other. After males had habituated to their territories for at least 1 week, we staged a series of encounters between pairs of males to encourage development of normal male behaviors (initially seven encounters, reduced to five after the first set of eight tests because this was sufficient to establish normal competitive behavior). Encounters were initiated by opening the link tunnel; from within the room, an observer then scored the number of times each male entered the other's enclosure, as well as all attacks, chases, threats, evasions, and attempts to flee. Each interaction continued either until we observed three successive aggressive interactions or until 15 min had elapsed. Males were allowed to interact a maximum of three times on any one day, although interactions were more usually spread over 1 week or more. After these encounters, we provided males with bedding from a mixture of randomly selected wild females to ensure that they were familiar with female odors.

Greater aggression in one male of a pair was associated with increased submissive behavior in the other male (homozygous male aggression versus heterozygous male evasion: $r_s = 0.595$, $p < 0.001$; vice versa: $r_s = 0.675$, $p < 0.001$). Males that were more aggressive also showed less submissive behavior themselves (among homozygous males: $r_s = -0.368$, $p = 0.009$; heterozygous males: $r_s = -0.295$, $p = 0.04$). The number of times a male initiated aggressive behavior was considered the most objective measure of male territorial behavior, and we converted this to a binary variable for analysis: If the heterozygous male showed a higher frequency of aggressive behaviors than did the homozygote, we assigned the pair a score of 1, and we assigned a score of 0 if the homozygous male was the more aggressive.

On the day before the female preference test commenced, we replaced the link tunnel in each pair of enclosures with a modified tunnel which contained a narrower (D × L: 26 mm × 135 mm) central Perspex tube, held in place with two horizontally placed brass rods that also served to further constrict its internal dimensions (to approximately 15 mm × 26 mm). The resulting opening was large enough to allow females passage through the tunnel but small enough to ensure that the males (which were much larger) remained in their own enclosures. Tunnel caps were left off overnight to allow males to scent mark the tunnel entrances.

### Female Preference

On the day before the full trial with males present, we allowed females to become familiar with the male enclosures and their scent marks in the absence of the males. This also allowed us to test female preference between the scent-marked male enclosures in the absence of the territory owners. Males were captured and temporarily returned to their home cages. The subject female was then placed in the closed link tunnel for 10 min before the tunnel was opened and the female allowed into the enclosures for 7 ± 0.1 hr. Females were recaptured and returned to their group cages overnight, and males returned to their respective territories.

The following day, we tested female preference for male territories with the owners present. Female subjects were again placed in the link tunnel for 10 min before being released into the enclosures for 78 ± 0.4 hr, comprising four dark and three light phases. At the end of the trial, females were recaptured and returned to their group cages.

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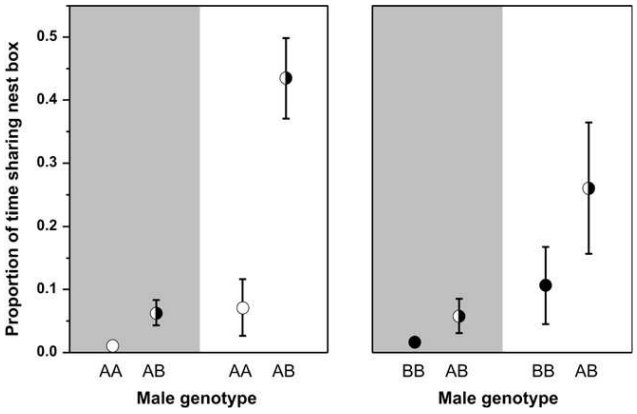


Figure S1. Preference for Nesting with MUP Heterozygous Males compared to Different MUP Homozygotes

The effect of male MUP genotype on the proportion of total time spent in the nest with each male during dark (shaded background) and light (unshaded background) phases (mean ± SE). Females spent longer with MUP heterozygous (half-filled circles) than with MUP homozygous males of either MUP type (MUP type AA: open circles; MUP type BB: filled circles). Although the effect appeared to be stronger when the homozygous male was of MUP type AA (derived from the BALB/c strain), there was no significant effect of homozygous MUP type on the magnitude of association time (repeated-measures GLM, interaction term: $F_{1,12} = 1.52$, $p = 0.24$). Similarly, in the test of MHC heterozygosity, the genotype of the MHC homozygote did not significantly affect female association time (interaction term: $F_{1,12} = 0.99$, $p = 0.34$).

### Male Heterozygosity and Dominance

We used a separate set of 11 pairs of F1 and F2 males (with heterozygosity at MUP and MHC held constant) to check for the expected relationship between genome-wide heterozygosity and dominance. Males were placed in standard mouse cages (internal area: 960 $cm^2$ × 13 cm h) that were divided by a central clear Perspex partition containing a single wire-mesh-covered opening (55 mm diameter) that allowed olfactory and visual, but not physical, contact between the two males. Several times per week, the central partition was removed, and the males were allowed to interact until either we observed one aggressive encounter with a clear winner, three aggressive interactions with no clear winner, or 10 min had elapsed. A consistently dominant male was identified in every pair but one after 16 to 25 days (14–20 interactions). The remaining pair was subsequently returned to the divided cages and established a stable hierarchy after an additional six interactions.

### Statistical Analysis

Sample sizes differ for different statistical tests because of occasional missing RFID data for males; in these cases, we were still able to analyze female behavior. All data were converted to proportion of total female records to account for rare missing records (when a mouse moving through an RFID or infrared sensor was not detected, revealed by a subsequent sensor record) and minor differences in total trial duration; proportions were then arcsine-square-root transformed prior to analysis. We analyzed the effect of male genotype on female behavior by using two-way repeated-measures general linear models: Time in each male's territory was modeled as a repeated measure for each female, and lighting phase was modeled as a second repeated measure. In all analyses, either the circadian-cycle phase was included as a variable or the light and dark phases were analyzed separately, because animals spent substantially more time active during the dark phase and combining these data leads to large variances. We tested all response variables and residuals from all models for departures from normality.

Table S1. Breeding Program

| | MUP and H2 Genotypes | | | |
|---|---|---|---|---|
| Generation | $MUP^{AA}$ | $MUP^{AB}$ | $MUP^{BB}$ | Heterozygosity at Genetic Background |
| Parental | $MUP^{AA}H2^{bb}$ | | $MUP^{BB}H2^{dd}$ | 0% |
| F1 | | $MUP^{AB}H2^{bd}$ | | 100% |
| F2 | $MUP^{AA}H2^{bb}$ | $MUP^{AB}H2^{bb}$ | $MUP^{BB}H2^{bb}$ | 50% |
| F2 | $MUP^{AA}H2^{bd}$ | $MUP^{AB}H2^{bd}$ | $MUP^{BB}H2^{bd}$ | 50% |
| F2 | $MUP^{AA}H2^{dd}$ | $MUP^{AB}H2^{dd}$ | $MUP^{BB}H2^{dd}$ | 50% |

Subject males were derived from two highly inbred laboratory strains, BALB/c ($MUP^{AA}$, $H2^{bb}$) and C57BL/6 ($MUP^{BB}$, $H2^{dd}$); these were reciprocally paired to produce F1s that were genetically heterozygous at MUP, MHC (called *H2* in the mouse), and all other background loci that differ between the two parental strains. These F1s were in turn bred to produce F2s, at which time we simultaneously bred a second set of F1s to allow us to assign age-matched F1-F2 pairs. The F2 males were genotyped; MUP and MHC segregants were selected to produce male-male test pairs (see Table S2).

Table S2. Male Pairs for the Three Mate Choice Tests

| | Homozygous Male | | | Heterozygous Male | | | |
|---|---|---|---|---|---|---|---|
| Test Type | MUP | MHC | Background | MUP | MHC | Background | n trials |
| MUP | | | | | | | |
| | AA | bb | 50% | AB | bb | 50% | 1 |
| | AA | bd | 50% | AB | bd | 50% | 3 |
| | AA | dd | 50% | AB | dd | 50% | 2 |
| | BB | bb | 50% | AB | bb | 50% | 2 |
| | BB | bd | 50% | AB | bd | 50% | 4 |
| | BB | dd | 50% | AB | dd | 50% | 5 |
| MHC | | | | | | | |
| | AA | bb | 50% | AA | bd | 50% | 1 |
| | AA | dd | 50% | AA | bd | 50% | 4 |
| | AB | bb | 50% | AB | bd | 50% | 2 |
| | AB | dd | 50% | AB | bd | 50% | 3 |
| | BB | bb | 50% | BB | bd | 50% | 3 |
| | BB | dd | 50% | BB | bd | 50% | 3 |
| Background | | | | | | | |
| | AB | bd | 50% | AB | bd | 100% | 16 |

Within each pair, the genotype of the nontested genetic region (MHC, MUP, or genetic background) was held constant, while genetic heterozygosity at the other varied between the two members of the pair. In the MUP and MHC tests, all males were F2 stock and thus genetically heterozygous at random loci representing on average 50% of the genetic background. To test for female response to genetic heterozygosity across the rest of the genome, we paired F1 males with a MUP and MHC heterozygous F2. MUP and MHC were thus held constant in this test, while F2s had on average half the heterozygosity of F1s at background regions.

### Genotyping

MUP and MHC type of F2 mice were established by genotyping to identify haplotypes with microsatellite markers. Two MUP (D4NDS6 and D4MIT1164) and two MHC (D17MIT230 and D17MIT24) loci were sufficient to distinguish the four possible haplotypes. For full details of genotyping protocol, see [S5].

### Biochemistry

Protein concentrations of intact urine samples were determined with the Coomassie plus protein assay reagent kit (Pierce, United Kingdom). A standard curve was generated with a stock solution of bovine serum albumin (1 mg/ml diluted to the range 0–50 μg/ml with MilliQ water [Millipore, United Kingdom]). Urine samples were diluted 1:500 with MilliQ water and pipetted in duplicate to a 96-well microtiter plate, and 250 μl Coomassie reagent was added. Plates were read at 595 nm in a Labsystems iEMS Reader MF, and a standard curve and protein concentrations were calculated with Genesis 3.04 software (Life Sciences, United Kingdom).

We measured creatinine concentration in the urine to standardize for urine dilution; creatinine is a byproduct of muscle metabolism and is excreted at a constant rate by animals of similar muscle mass. Urine creatinine values were measured with a standard alkaline picrate assay (Sigma Chemicals, United Kingdom). Creatinine standards were generated from a stock solution of creatinine (3 mg/dl diluted to the range 0–30 μg/ml with MilliQ water). Each sample (100 μl of 1:100 dilution of urine) was pipetted in duplicate to a 96-well microtiter plate, and 150 μl of alkaline picrate reagent (5 ml picrate solution: 1 ml sodium hydroxide) was added. Plates were read in

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Table S3. Female Behavioral Response to Variation in Male Heterozygosity at MUP, MHC, and Genetic Background

| | Proportion of Female Time[a] | | | | | |
|---|---|---|---|---|---|---|
| | Dark Phase | | Light Phase | | | |
| Location, Time Period | Homozygous Male | Heterozygous Male | Homozygous Male | Heterozygous Male | F | p |
| **MUP** | | | | | | |
| Territory, day 0[b] | 45.6 ± 4.5 | 53.0 ± 4.4 | – | – | $F_{1,15} = 0.11$ | 0.740 |
| Territory, day 1–4[b] | 46.3 ± 5.2 | 50.0 ± 5.4 | 38.9 ± 7.9 | 57.2 ± 8.5 | $F_{1,15} = 0.55$ | 0.468 |
| Nest, day 0 | 7.5 ± 1.9 | 13.1 ± 3.8 | – | – | $F_{1,14} = 0.83$ | 0.377 |
| Nest, day 1–4 | 9.9 ± 1.9 | 20.6 ± 3.8 | 15.8 ± 4.6 | 38.1 ± 7.4 | $F_{1,15} = 4.12$ | 0.060 |
| Nest with male, day 1–4 | 1.3 ± 0.4 | 6.0 ± 1.8 | 9.4 ± 4.2 | 32.3 ± 7.2 | $F_{1,13} = 5.95$ | 0.030 |
| **MHC** | | | | | | |
| Territory, day 0[b] | 54.9 ± 4.7 | 43.7 ± 4.6 | – | – | $F_{1,13} = 1.5$ | 0.242 |
| Territory, day 1–4[b] | 52.8 ± 5.4 | 42.4 ± 5.6 | 53.8 ± 7.5 | 43.5 ± 7.7 | $F_{1,15} = 0.74$ | 0.402 |
| Nest, day 0 | 8.7 ± 2.1 | 4.3 ± 1.6 | – | – | $F_{1,13} = 3.89$ | 0.070 |
| Nest, day 1–4 | 14.6 ± 3.2 | 12.0 ± 3.1 | 24.5 ± 6.6 | 20.7 ± 5.3 | $F_{1,15} = 0.14$ | 0.710 |
| Nest with male, day 1–4 | 3.8 ± 1.7 | 2.7 ± 0.9 | 16.8 ± 5.4 | 17.1 ± 4.6 | $F_{1,13} = 0.02$ | 0.965 |
| **Genetic Background** | | | | | | |
| Territory, day 0[b] | 58.6 ± 5.3 | 41.1 ± 5.3 | – | – | $F_{1,15} = 2.7$ | 0.124 |
| Territory, day 1–4[b] | 43.2 ± 4.0 | 52.2 ± 4.4 | 45.1 ± 6.0 | 50.7 ± 6.5 | $F_{1,15} = 0.57$ | 0.460 |
| Nest, day 0 | 16.6 ± 5.5 | 8.2 ± 2.2 | – | – | $F_{1,15} = 1.22$ | 0.286 |
| Nest, day 1–4 | 14.6 ± 2.9 | 19.9 ± 3.5 | 25.6 ± 5.1 | 26.2 ± 5.7 | $F_{1,15} = 0.18$ | 0.678 |
| Nest with male, day 1–4 | 2.1 ± 0.6 | 1.6 ± 0.5 | 15.5 ± 3.5 | 15.3 ± 3.8 | $F_{1,14} = 0.05$ | 0.827 |

Data are shown for the proportion of total time females spent within each male's territory, time in each nest ignoring male presence or absence, and time coinhabiting the nest with the male. Analyses are two-way repeated-measures general linear models, with male heterozygosity at the test region as one repeated measure (to ensure appropriate pairing within female) and lighting phase as the other (data normalized by arcsine square-root transformation for analysis; untransformed data shown in table). The effect of lighting phase was significant in all models, as a result of the greater use of nest boxes during the light phase of the circadian cycle. Day 0 is day before main trial, with the female alone in the territory. During the main trial (day 1–4), both males were present in their territories.

[a] Sample sizes differ between measures (see Statistical Analysis).

[b] Values for total time in territory do not add up to 100% because this measure excludes female time in the link tunnel.

a Labsystems iEMS Reader MF at 492 nm, a standard curve was produced with Genesis software, and urine sample creatinine concentrations were calculated by interpolation. These values were used to calculate a protein:creatinine ratio for each urine sample.

Isoelectric focusing was used to separate MUPs by charge. We used precast, narrow-range (pH 4.2–4.9) immobilized pH gels (Pharmacia, United Kingdom) rehydrated in 15% glycerol and 2.5% ampholine (pH 3.5–9.5) and run on a Multiphor flatbed elecrophoresis system and MultiTemp III thermostatic circulator (Pharmacia, United Kingdom). Urine samples were standardized to 1 mg/ml with MilliQ water according to initial protein concentration, and 5 μl of the diluted sample applied to the gel with sample application pieces. Samples were drawn into the gel for 200 Vh, and after removal of the application pieces, the gel was focused for 14.8 KVh at 10°C. The gel was fixed in 20% (w/v) trichloroacetic acid for 1 hr, rinsed in a destain solution (acetic acid:methanol:MilliQ water, 5:30:65 v/v/v) for 20 min, stained in 0.02% (w/v) Coomassie Brilliant Blue with 0.1% (w/v) copper sulfate for 15 min, destained for 7.5 hr twice, and preserved in 12.5% glyverol (v/v) for 1 hr.

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