Supporting Information

Nelson et al. 10.1073/pnas.1310427110

SI Materials and Methods

Animal Stocks and the Effects of Domestication. Mice were derived from a cross between wild-caught mice and MHC-congenic mice carrying five known haplotypes (C57BL/10SnJ-H2b, B10.D2-H2d, B10.M-H2f, B10.BR-H2k, and B10.Q-H2q) obtained from The Jackson Laboratory, where wild-derived MHC haplotypes were eliminated by selective breeding (1). This "congenic/wild" strain is genetically diverse, but has well-characterized MHC haplotypes, and allowed us to control for MHC-mediated mating behaviors during direct competition and mate-choice experiments (2). This strain had bred within a laboratory environment for 10 generations before being introduced to sociality and promiscuous breeding in seminatural enclosures.

To address how captivity and breeding with inbred laboratory strains may have affected this congenic/wild strain, we first compared male and female weaning weights from three populations: the first generation of laboratory-bred wild mice (3); the thirteenth generation of laboratory-bred wild mice; and the thirteenth generation of congenic/wild mice. Here, we found evidence for considerable weight gain as a result of captivity: thirteenth generation wild and wild/congenic mice were found to be several grams heavier than the first generation wild mice, with congenic/wild mice having the greatest weight. Pairwise comparisons between all strains were significant in both male and female mice (Bonferroni's multiple correction test, $P < 0.05$) (Fig. S1).

Next, to address whether captivity may have affected the social behavior of the wild/congenic strain, we compared PIT-tag data from independent seminatural enclosure populations of the thirteenth generation of laboratory wild mice ($n = 3$), the thirteenth generation of wild/congenic mice ($n = 3$), and an inbred laboratory strain (BALB/d; $n = 1$). Each enclosure consists of an average of 10 males and 20 females and contains four optimal territorial sites. Social dominance is established by exclusive representation at the optimal sites (2). Here, we measured the average number of socially dominant males and females found at each optimal site for each strain. Results indicate that wild and congenic/wild mice behave similarly (Fig. S2). For both wild and congenic/wild populations, there are 0.75 dominant males per optimal territorial site whereas there are only 0.25 dominant males per site for the inbred laboratory strain, who display the abnormal behavior of visiting all territories. The number of dominant congenic/wild and wild females at each optimal territorial site is 1.92 and 2.00, respectively whereas there are zero dominant BALB/d females per site, who also display the abnormal behavior of frequently visiting multiple territories (Fig. S2).

Together with our previous reports on the seminatural territorial behaviors of first-generation wild mice (3) and the fifth-generation congenic/wild mice (1), these data suggest that the social ecology of the congenic/wild mouse strain studied here is normal. In addition, the effects of adaptation to captivity and the laboratory environment have exerted a strong effect on body mass, and other studies have reported similar effects (4), suggesting that several physiological systems are disrupted during domestication. Although body weight did not contribute to any of the differences here or in the initial study (2), it is possible that other systems disrupted by captivity contributed to the effects reported here.

Testosterone. Two blood samples from each promiscuous-line ($n = 15$) and monogamous-line male ($n = 15$) were collected by submandibular punctures with 4-mm Goldenrod lancets (Medipoint Inc), first from sexually naive, individually housed males at 4 mo of age. One month later, estrus females (identified by vaginal lavage cytology) were each placed on top of male cage-lids (with food and water removed) and enclosed with a microisolator filter top, thus preventing variation in mating behavior across trials. After a 30-min exposure, the second blood sample was collected. Samples were kept on ice and centrifuged at 4 °C for 15 min at 14,006 × g; the supernatant was collected and frozen (−70 °C). Frozen samples were shipped overnight to Ohio State University (Columbus, OH), and analyzed by RIA. Plasma testosterone concentrations were determined using a Diagnostic Systems Laboratories ^{125}I double Ab kit. Detection range of the assay was 0.2–25 ng/mL. To test for differences across time points, we used repeated-measures ANOVA with testosterone as the dependent variable and time-point and trial number as effects. Within time points, we compared testosterone levels using one-way ANOVA.

Urine Collection, Protein Assays, and Creatinine. Urine was collected from F_4 individually housed, cage-born promiscuous-line and monogamous-line males (~180 d of age; $n = 15$ of each treatment). Urine from F_5 males was collected at postnatal day 21 (with brother siblings; promiscuous line $n = 17$; monogamous line $n = 13$); day 60 (with brother siblings; $n = 23$ of each treatment); and day 110 (housed individually; $n = 20$ of each treatment). Urine was collected by holding mice by the scruff and tail base over a clean Plexiglas sheet and immediately transferred to a microcentrifuge tube, flash frozen in liquid nitrogen, and stored at −70 °C. Following a 1:20 dilution of whole urine, total urinary protein concentration (of which >95% is MUPs) (5, 6) was determined with the Bradford Assay (Pierce) according to the manufacturer's instructions. Whole urine was used to measure creatinine using a colorometric assay based on Jaffe's basic picrate method (Stanbio Liquicolor Kit), according to the manufacturer's instructions. One-way ANOVA was used to compare treatment effects on MUP expression, creatinine excretion, and normalized MUP/creatinine ratios.

Single-Generation Reciprocal-Breeding Experiment. Mice were randomly assigned to competitive (C) and noncompetitive (NC) treatments, avoiding cousin-level (and higher) inbreeding. NC males and females experienced enforced monogamy ($n = 23$ breeding cages) for 8 wk and then were individually housed for 3 wk, during which time any pregnant females reached parturition and their pups were killed. Simultaneously, C males ($n = 20$) and females ($n = 40$) experienced competition in two seminatural enclosures (following the same protocol as for promiscuous-line breeders) (2) for 8 wk, at which time females were removed, individually housed for 3 wk, and any pups were killed. During the final 3 wk, C males remained in the enclosures with "placeholder" females. At the end of the exposure, males and females of both treatments were assigned to a monogamous breeding cage in a randomized design. After 8 d, the males were removed from the breeding cage. Offspring were weaned at 3 wk of age, and urine from adolescent males was collected at 70 d of age. Adult sons were individually housed at 21–22 wk of age, and their urine was collected after 4 d. A general linear model—with urinary protein as the dependent variable and maternal treatment, paternal treatment, and maternal × paternal interaction as independent variables—was used to assess transgenerational effects on MUP output. Subsequently, a general linear model was used to partition several sources of potential variation. Urinary protein was modeled as the dependent variable, and maternal treatment, paternal treatment, maternal × paternal treatment

interaction, and creatinine concentration were added as independent variables. Litter number was added as a random effect to account for the effects of common litter.

Reverse Transcription Quantitative PCR. Liver RNA was extracted from F_4 promiscuous-line ($n = 8$) and monogamous-line ($n = 10$) males using an RNeasy extraction kit (Qiagen). RNA integrity was verified by an average optical density 260/280 nm absorption ratio of 1.97. RNA concentrations were standardized by diluting all samples to ∼20 ng/μL in HPLC water. Reactions were optimized at a final volume of 10 μL and performed with the Lightcycler RNA SYBR Green Reaction Kit (Roche). Cycling parameters included an initial reverse-transcription step to convert RNA to cDNA (per the manufacturer's instructions), an initial denaturation 94 °C for 30 s, 40 PCR cycles (5 s at 95 °C, 10 s at 65 °C, 19 s at 72 °C), a melt analysis measuring fluorescence intensity every 0.1 °C increase from 65 °C to 95 °C, and a final cooling step down to 40 °C. The reference gene *Gapdh* was used as a housekeeper comparison, and all reported gene-expression data are target/*Gapdh* ratio values. Subsets of qPCR products were cloned with pGEM-T cloning kits (Promega) and sequenced to confirm amplicon identity. A one-way ANOVA was used to test for treatment effects.

Bisulfite Sequencing. Liver and tail DNA (2 μg) from promiscuous-line ($n = 4$) and monogamous-line ($n = 4$) males was sodium bisulfite converted as previously described (7). Seminested PCR was used to amplify four CpGs in the 5′ untranslated region and the first exon of MUP 11 using primers designed by Methprimer (primers in Table S1). The seminested product was 223 bp. Cycling parameters included an initial denaturation at 94 °C for 16 min followed by 40 rounds of denaturation at 94 °C for 40 s, annealing for 1 min, and extension at 72 °C for 1 min. A final extension step was carried out at 72 °C for 5 min. PCR fragments were isolated from agarose gels using spin columns (Qiagen) and further purified with phenol-chloroform extraction and ethanol precipitation. The bands were cloned with pGEM-T cloning kits (Promega) as per the manufacturer's instructions. Colonies were selected on agar plates containing ampicillin and plasmids isolated with miniprep kits (Qiagen). Clones were sequenced using the M13R primer. Individually sequenced clones were analyzed with QUMA (RIKEN Institute). To avoid the potential bias from sampling the same locus more than once, the criterion was applied that each clone from an individual mouse must differ by at least one nucleotide to be included in the analysis. Any clones with less than 90% conversion rate were excluded. To test for statistical differences in methylation, we used Fisher's exact test.

Chromatin Immunoprecipitation. Candidate transcription factors were identified using the software PROMO (8). Livers from two wild-derived mice from our colony were minced, rinsed with PBS, and cross-linked in 1% (wt/vol) formaldehyde at room temperature for 15 min. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M with a 10-min incubation at room temperature. Tissue was homogenized in 8 mL of cold PBS with a Dounce homogenizer, recovered by centrifugation at 1,000 × *g* for 3 min at 4 °C and rinsed with cold PBS. Hepatocytes were resuspended in 8 mL of cell lysis buffer [50 mM Hepes-KOH, pH 8.0, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.25% (vol/vol) Triton X-100, 0.5% (vol/vol) Nonidet P-40 plus 1.5× protease inhibitors], homogenized again, and pelleted by centrifugation at 1,000 × *g* for 5 min at 4 °C. Lysed cells were resuspended and rinsed with 10 mL of nuclei wash buffer [10 mM Tris·HCl, pH 8, 200 mM NaCl, 1 mM EDTA, and 1% (wt/vol) SDS plus 1.5× protease inhibitors] for 3 min at 4 °C and pelleted by centrifugation at 700 × *g* for 3 min at 4 °C. Nuclei were resuspended in 10 mL of nuclei lysis buffer [50 mM Tris·HCl, pH 8, 100 mM NaCl, 10 mM EDTA, and 1% (wt/vol) SDS plus 1.5× protease inhibitors]. Chromatin was sheared by sonicating (Misonix) 10–20 times to an average shear length of 500 bp. Input DNA (i.e., no-antibody) was prepared by adding 50 μL of sonicate to 100 μL of TE and 200 mM NaCl to reverse cross-links and incubated at 65 °C overnight.

Eluate DNA (i.e., USF-bound) was prepared by binding 100 μL of Dynabeads (Invitrogen) to 10 μg of anti-USF1 antibody (sc-229; Santa Cruz Biotechnology) in dilution buffer (15 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mg/mL 1 BSA) and incubated at 4 °C on a rotator overnight. The sonicate was diluted 10-fold in dilution buffer, precleared with 50 μL of Dynabeads for 1 h, and transferred to bead–antibody complexes for overnight immunoprecipitation at 4 °C. Beads were isolated on a magnetic stand and washed in succession with 800 μL of the following wash buffers: WB140 [20 mM Tris·HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) Na deoxycholate]; WB500 (same as WB140 but with 500 mM NaCl); WBLiCl [10 mM Tris·HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% (vol/vol) Nonidet P-40, 0.5% (wt/vol) Na deoxycholate]; and TE. Cross-links were then reversed in 200 mM NaCl. Input and eluate DNA was purified with phenol-chloroform-isoamyl (25:24:1, pH 8; Invitrogen) and Qiagen PCR Purification.

For quantitative PCR, we used a 1:50 dilution of chromatin immunoprecipitation (ChIP) eluate (each sample run in triplicate) and iQ SYBR Green Supermix (Bio-Rad) in a total volume of 20 μL. ChIP input DNA was serially diluted to generate a standard curve for each primer pair. We analyzed PCR results with iCycler (Bio-Rad). Primers were taken from published literature as indicated (Table S1) or designed with Primer3.

Odor-Preference Assay. High- and low-concentration odor cages (dimensions: 18 in × 12 in × 6 in) were connected to a neutral chamber by 6-in lengths of PVC pipe (2-in diameter). Infrared, black and white cameras were mounted above each of three arenas. We conducted 12 trials, each lasting 30 min. Video was captured and analyzed with a Timescience recording system. Urine (>250 μL) was collected from 12 males, which were unrelated to the test females. High molecular weight (HMW) and low molecular weight (LMW) protein fractions were separated using 10-kDa molecular weight cutoff Amicon Ultra centrifugal filters (Millipore). The HMW and LMW fractions were resolved on 15% one-dimensional SDS/PAGE as described (9). The only bands observed in the HMW fractions were MUPs (Fig. 2*A*) (10). Protein concentrations of each fraction were determined with the Coomassie plus protein assay reagent kit (Pierce) and HMW fractions were >100 mg/mL. From each urine sample, we prepared a high (20 mg/mL) and low (3 mg/mL) MUP concentration version by aliquoting HMW concentrate back to two equal volumes of LMW filtrate to achieve either the 20 mg/mL or 3 mg/mL final MUP concentration samples. Mimicking urinary scent marks, 5-μL aliquots of high and low concentration samples were pipetted onto a grid of 18 spots on two separate filter papers (5 in × 2.5 in). Papers were taped vertically to the wall most proximal to the tunnel entrance. Vertical placement of scent marks makes for easier identification of sniffing behaviors and is a natural site of scent marks. The position of the high or low MUP concentration scent marks was balanced between trials to prevent any side preference by females.

Odor preference was assessed by measuring time females spent in direct contact (nose within 1 in of scent marks) with the scent marks. Females had to enter the cage with all four feet to make direct contact with the scent marks. Comparisons of proportions were made using nonparametric paired *t* tests (Wilcoxon signed-ranks). To compare sniffing order and the amount of time sniffing, and whether the slopes of the lines (i.e., high vs. low concentration) were different, linear regression was used.

1. Ilmonen P, et al. (2007) Major histocompatibility complex heterozygosity reduces fitness in experimentally infected mice. *Genetics* 176(4):2501–2508.
2. Nelson AC, Colson KE, Harmon S, Potts WK (2013) Rapid adaptation to mammalian sociality via sexually selected traits. *BMC Evol Biol* 13(1):81.
3. Meagher S, Penn DJ, Potts WK (2000) Male-male competition magnifies inbreeding depression in wild house mice. *Proc Natl Acad Sci USA* 97(7):3324–3329.
4. Miller RA, Harper JM, Dysko RC, Durkee SJ, Austad SN (2002) Longer life spans and delayed maturation in wild-derived mice. *Exp Biol Med (Maywood)* 227(7):500–508.
5. Robertson DH, Cox KA, Gaskell SJ, Evershed RP, Beynon RJ (1996) Molecular heterogeneity in the Major Urinary Proteins of the house mouse Mus musculus. *Biochem J* 316(Pt 1):265–272.
6. Beynon RJ, et al. (2002) Polymorphism in major urinary proteins: Molecular heterogeneity in a wild mouse population. *J Chem Ecol* 28(7):1429–1446.
7. Arnaud P, et al. (2003) Conserved methylation imprints in the human and mouse GRB10 genes with divergent allelic expression suggests differential reading of the same mark. *Hum Mol Genet* 12(9):1005–1019.
8. Messeguer X, et al. (2002) PROMO: Detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 18(2):333–334.
9. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
10. Roberts SA, et al. (2010) Darcin: A male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol* 8:75.

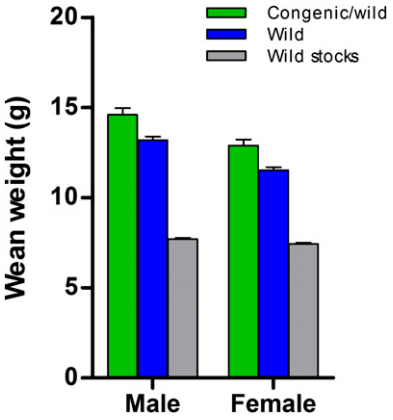

Fig. S1. Effects of captivity on weaning weight among three strains of mice. The congenic/wild and wild mice weights are from the 13th generation of captivity. Wild-stock weights are from the first generation of captivity. Within-sex comparisons of each strain are all significantly different (Bonferroni's multiple correction test, $P < 0.05$).

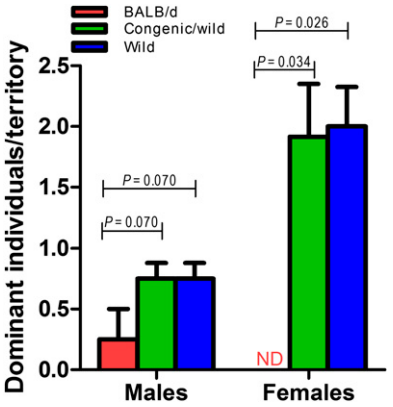

Fig. S2. Territorial behavior among three strains of mice. PIT-tag data from independent seminatural enclosures were used to estimate the number of dominant males and females per optimal nesting territory. Student *t* test *P* values shown for each comparison. ND, none detected.

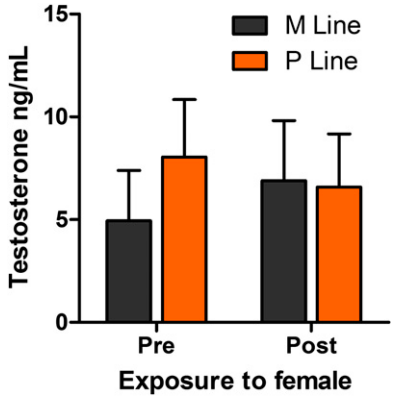

Fig. S3. Circulating testosterone in promiscuous-line and monogamous-line males before and after exposure to an estrus female. Means and SEs are repeated measures from 12 males.

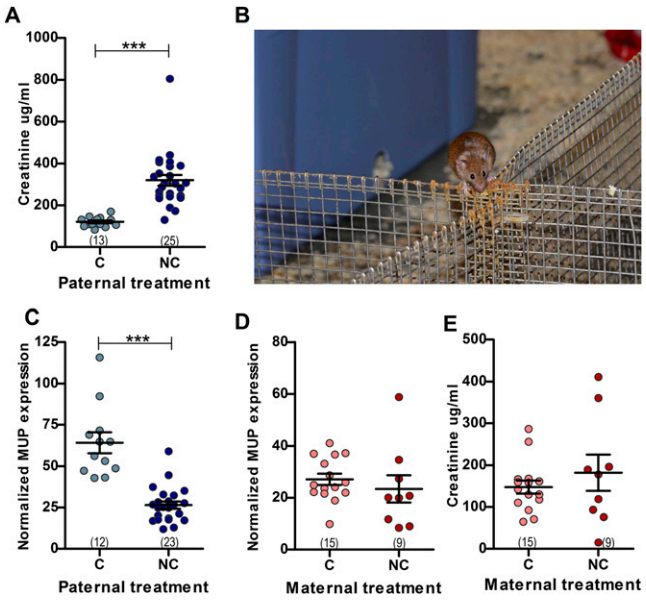

Fig. S4. Direct effects of competition (C) and noncompetition (NC) on urinary MUP expression and creatinine excretion. C fathers had lower urinary creatinine excretion (*A*) and higher creatinine-normalized MUP expression (*C*) than NC fathers. (*B*) Urinary scent marks on territorial boundaries in C enclosures, visible as a brown paste at the intersection of fences. C and NC mothers had equivalent creatinine-normalized MUP expression (*D*) and creatinine excretion (*E*). Means ± SEM, GLM, $***P < 0.0001$.

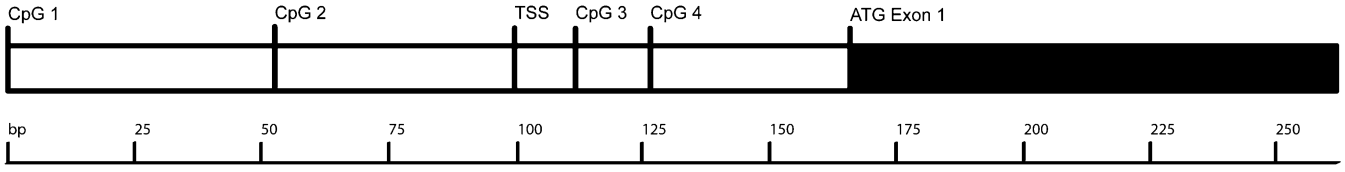

Fig. S5. MUP 11 gene structure and location of CpG sites. CpG site 4 is 27 bp 3′ of the transcriptional start site (TSS), 39 bp 5′ of the start codon of exon 1, and contains the putative binding site of USF1.

Table S1. Primer sequences for rt-qPCR, bisulfite sequencing, and ChIP

Experiment	Primer name	Lab ID	Locus description	Sequence
Real-time qPCR	Mup2-F (1)	WP643	Mup2, one of the most abundant	5′-ATTAATGGGGAATGGCATACTA-3′
	Mup2-R (1)	WP644	variants in mice	5′-GGATTCCATGCTCCTCACAT-3′
	Mup3-F (1)	WP646	Mup3, one of the most abundant	5′-GCAAGTGTAATCATTTATTGAACAGG-3′
	Mup3-R (1)	WP645	variants in mice	5′-ATGGAGCTCTATGGCCGAG-3′
	Mup11-F (2)	WP691	Mup11 forward primer	5′-ATGAAGATGCTGTTGCTG-3′
	Mup15-F (2)	WP693	Mup15 forward primer	5′-ATGCTGCTGCTGCTGTGT-3′
	Mup18-F (2)	WP688	Mup15 forward primer	5′-GAAGAATCTAGTTCTATG-3′
	Mup25-F (2)	WP686	Mup25 forward primer	5′-GAAGAATCTAGTTCTATG-3′
	Mup13,14,17-F (2)	WP692	Forward primer targeting a conserved sequence between 3 Mup RNAs	5′-ATGCTGTTGCTGCTGTGT-3′
	Mup3,7,9,12,16-F (2)	WP687	Forward primer targeting a conserved sequence between 5 Mup RNAs	5′-ATGAAGATGCTGCTGCTG-3′
	Mup2-18-R (2)	WP699	Reverse primer for Mup2–Mup18	5′-TCA TTC TCG GGC CTT GAG-3′
	Mup25-26-R (2)	WP697	Reverse primer for Mup25 and Mup26	5′-TCATTCTCGGGCCTCGAG-3′
	Darcin-F	WP744	Based on ref. 3. Designed in	5′-GCATAATAGTATACCATTCCCCAT-3′
	Darcin-R	WP745	Primer3.	5′-TCATTCTCGGGCCTCAAG-3′
	Darcin-R (2)	WP745b		5′-TCATTCTCGGGCCTCAAG-3′
	Gapdh-F	WP612	Housekeeping reference transcript	5′-TGTTGCTGTAGCCGTATTCA-3′
	Gapdh-R	WP613		5′-CTGGAGAAACCTGCCAAGTA-3′
	IGF-F (4)	WP729	Proxy for the activity of the	5′-CTTCAACAAGCCCACAGGCTAT-3′
	IGF-R (4)	WP728	GH-JAK-STAT5b transduction pathway	5′-GCTCCGGAAGCAACACTCAT-3′
Bisulfite sequencing	Mup promoter-F		Bisulfite sequencing of four CpGs	5′-TTTGAGTTAGAAGTTATTATTTAGTGATTA -3′
	Mup promoter-R		in the MUP promoter using seminested	5′-TAACAAAAATAAAAAAAACCCATAC -3′
	Mup Seminested-F		PCR	5′-AGGGTTAGTTTTAAATATAGTAGTAATAGT-3′
ChIP	GPAT-F (5)		ChIP positive control I	5′-CCCTTTAACTGGGAGAGCAGAG-3′
	GPAT-R			5′-TTTTGTTCAGGGTGATCTTTTGCC-3′
	LDLR-F (6)		ChIP positive control II	5′-CGCCGCGTTTTATATACATTTCTCG-3′
	LDLR-R			5′-CAAAGCAAAACAGAAGTATGCGAAGC-3′
	Gene desert-F		ChIP negative control	5′-AGCGCTCAGCACAGAATTGC-3′
	Gene desert-R			5′-CACAGTGAAATCAACAGCCATGC-3′
	Mup promoter-F		Mup promoter region with putative	5′-ACATTCCACAAAGCCTGACAGAGG-3′
	Mup promoter-R		USF-1 binding site	5′-GCAGCAGCAGCATCTTCATTTTG-3□

1. Stopková R, Stopka P, Janotová K, Jedelský PL (2007) Species-specific expression of major urinary proteins in the house mice (Mus musculus musculus and Mus musculus domesticus). *J Chem Ecol* 33(4):861–869.
2. Logan DW, Marton TF, Stowers L (2008) Species specificity in major urinary proteins by parallel evolution. *PLoS ONE* 3(9):e3280.
3 Roberts, SA et al. (2010) Darcin: A male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol* 8:75.
4. Davey HW, et al. (2001) STAT5b is required for GH-induced liver IGF-I gene expression. *Endocrinology* 142(9):3836–3841 10.1210/en.142.9.3836.
5. Yoshida M, et al. (2009) Identification of cis-acting promoter sequences required for expression of the glycerol-3-phosphate acyltransferase 1 gene in mice. *Biochim Biophys Acta* 1791(1):39–52.
6. Latasa MJ, Griffin MJ, Moon YS, Kang C, Sul HS (2003) Occupancy and function of the -150 sterol regulatory element and -65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. *Mol Cell Biol* 23(16):5896–5907.

Table S2. Competition (C) driven maternal effect on litter size (n = 32 litters)

Term	Estimate	Std error	DFDen	t Ratio	P value
Intercept	7.41	0.320	28	23.18	<0.0001
Maternal (C)	0.816	0.320	28	2.55	0.017
Paternal (C)	−0.105	0.320	28	−0.33	0.744
Maternal (C) x paternal (C)	0.069	0.320	28	0.22	0.831

Table S3. Variance (Var) components of male MUP expression due to maternal and paternal effects, and litter effects (i.e., "birthcage") (n = 77)

Effect	Var ratio	Var component	% of total
Birthcage	0.253	192,799.9	15.88
Father treatment	0.111	84,768.3	6.98
Mother treatment	0.229	175,118.2	14.42
Residual		761,430.4	62.72
Total		1,214,117	100.000

Table S4. Competition (C) driven maternal and paternal effects on creatinine excretion in sons

Term	Estimate	Std error	DFDen	t Ratio	P value
Sibling housed (age 70 d) (n = 81)					
Intercept	336.94	13.95	25.28	24.15	<0.0001
Father treatment (C)	23.49	13.95	25.28	1.68	0.105
Mother treatment (C)	28.94	13.95	25.28	2.07	0.048
Father treatment x mother treatment (C)	23.11	13.95	25.28	1.66	0.110
Individually housed (age 170 d) (n = 78)					
Intercept	563.43	21.26	14.48	26.49	<0.0001
Father treatment (C)	−3.30	21.26	14.48	−0.16	0.879
Mother treatment (C)	−15.13	21.26	14.48	−0.71	0.488
Father treatment (C) x mother treatment (C)	17.78	21.26	14.48	0.84	0.417