Published in final edited form as:

J Evol Biol. 2015 June ; 28(6): 1213–1224. doi:10.1111/jeb.12643.

Protein pheromone expression levels predict and respond to the formation of social dominance networks

Adam C. Nelson^{1,2,4,*}, Christopher B. Cunningham^{1,3,4,*}, James S. Ruff¹, and Wayne K. Potts¹

Adam C. Nelson: adamnelson@fas.harvard.edu; Christopher B. Cunningham: cbc83@uga.edu; James S. Ruff: j.ruff@utah.edu; Wayne K. Potts: potts@biology.utah.edu

¹University of Utah, Department of Biology, Salt Lake City, UT 84112

²Harvard University, Department of Molecular and Cellular Biology, Cambridge, MA 02144

³University of Georgia, Department of Genetics, Athens, GA 30602

Abstract

Communication signals are key regulators of social networks, and are thought to be under selective pressure to honestly reflect social status, including dominance status. The odors of dominants and nondominants differentially influence behavior, and identification of the specific pheromones associated with, and predictive of, dominance status is essential for understanding the mechanisms of network formation and maintenance. In mice, major urinary proteins (MUPs) are excreted in extraordinary large quantities and expression level has been hypothesized to provide an honest signal of dominance status. Here, we evaluate whether MUPs are associated with dominance in wild-derived mice by analyzing expression levels before, during, and after competition for reproductive resources over three days. During competition, dominant males have 24% greater urinary MUP expression than nondominants. The MUP darcin, a pheromone that stimulates female attraction, is predictive of dominance status: dominant males have higher darcin expression before competition. Dominants also have a higher ratio of darcin to other MUPs before and during competition. These differences appear transient, because there are no differences in MUPs or darcin after competition. We also find MUP expression is affected by sire dominance status: socially naive sons of dominant males have lower MUP expression, but this apparent repression is released during competition. A requisite condition for the evolution of communication signals is honesty, and we provide novel insight into pheromones and social networks by showing that MUP and darcin expression is a reliable signal of dominance status, a primary determinant of male fitness in many species.

Keywords

Chemical signaling; competitive ability; male-male competition; sexual selection

Corresponding Author: Adam Nelson, Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Ave, Cambridge, MA 02138, 801-860-1976.

⁴Corresponding authors.

*Contributed equally to this study.

Data Availability: The data will be deposited into public repository (such as, Dryad) upon acceptance of the manuscript.

Introduction

Communication signals are a central feature of social networks. Signals are used to structure and regulate social network dynamics, and communicate information to conspecifics such as kinship (Cheetham et al., 2007), social status (Diep & Westneat, 2013), and health or vigor (Saks et al., 2003) through many different sensory modalities. Theory predicts that the evolution of signals is driven by a combination of signaling costs and physiological constraints, and as a result signals are thought to be reliable and honest indicators of specific traits (Holman, 2012). Thus, some signals are thought to accurately reflect social competitive ability, including roaring in red deer (Clutton-Brock & Albon, 1979), color patches of male red-winged blackbirds (Peek, 1972), possession of a tail in female side-blotched lizards (Fox et al., 1990), and pheromone profiles in the American cockroach (Moore et al., 1997). Identifying the temporal associations between social signals and social status provides an important first step for elucidating how signals influence social networks and provides of point of entry for understanding the evolutionary forces shaping social signals.

In many animals, including mice, social dominance networks are established through competition for reproductive resources that attract high quality mates and reproductive success is greatly influenced by dominance status, or the capacity to monopolize resources through repeatedly winning agonistic encounters (Bronson, 1979, DeFries & McClearn, 1970, Dewsbury, 1982, Cunningham et al., 2013, Ellis, 1995). Communication through urinary scent marking facilitates the stability of dominance status (Rich & Hurst, 1998, Rich & Hurst, 1999, Garratt et al., 2012). Dominant males urine mark and countermark more than nondominants (Desjardins et al., 1973, Drickamer, 2001), and females prefer males whose territory contains only his marks, or males who reliably countermark competitors (Rich & Hurst, 1998, Rich & Hurst, 1999). A primary determinant of social dominance is the status of the parents (Holekamp & Dloniak, 2009), and we previously demonstrated a high level of additive genetic variance for dominance status in wild-derived mice (Cunningham et al., 2013).

Identification of pheromones underlying dominance status is essential to understanding the signaling mechanisms underlying social network formation. Male mice produce a number of urinary volatiles and proteins that elicit social and sexual behavioral responses in conspecifics. The androgen-dependent volatile pheromone 2-sec-butyl-4,5-dihydrothiazole (SBT) stimulates females to sniff male scents, increases neurogenesis in the female brain, and possibly underpins the estrus inducing effect of male urine (Koyama et al., 2013, Jemio et al., 1985). Consistent with a potential role in dominance status, urinary SBT concentration is higher in aggressive males (Harvey et al., 1989). The protein component of mouse urine is comprised almost exclusively of major urinary proteins (MUPs). MUPs are encoded by roughly twenty linked loci and are integral to social communication (Mudge et al., 2008) by providing a signature of genetic identity and relatedness (Kaur et al., 2014, Sherborne et al., 2007, Cheetham et al., 2007). MUPs bind and slowly release many volatile urinary compounds (Hurst et al., 1998); the male-expressed MUP darcin is the primary carrier of SBT (Armstrong et al., 2005), and darcin by itself influences formation of social memory, particularly in females (Roberts et al., 2012, Roberts et al., 2010). MUPs are also

implicated in aggression: adult male urine swabbed on the back of castrated male intruders strongly elicits aggression from intact residents, and MUPs (including darcin) are sufficient to promote this effect (Chamero et al., 2007).

MUPs, including darcin, are candidate pheromone signals underlying dominance status in mice. MUPs are detected by vomeronasal neurons that express the G-protein $G\alpha_o$ subunit and Vmn2r receptors (Chamero et al., 2007). MUPs are excreted in extraordinarily large quantities and exhibit sexual dimorphism, with males expressing up to eightfold greater amounts than females (Stopkova et al., 2007, Cheetham et al., 2009). Because MUPs represent irretrievable protein loss, it has been hypothesized that expression is costly and therefore only males in good condition (i.e., dominant males) can afford high expression; in turn, expression level might be an honest signal of quality and competitive ability (Garratt et al., 2012, Beynon & Hurst, 2003, Garratt et al., 2011, Gosling et al., 2000, Stopkova et al., 2007, Cheetham et al., 2007, Janotova & Stopka, 2011). MUPs may therefore be a condition-dependent “handicap” signal, where males can vary signal intensity but the fitness cost of expression is greater for low quality males, thus ensuring signal honesty (Grafen, 1990, Zahavi, 1975). Alternatively, MUPs may not be costly to produce but nevertheless serve as an “index” of social status because expression level is causally linked to determinants of quality, such as body size; here, signal honesty is maintained because it cannot be faked (Maynard Smith & Harper, 1995). Consistent with these models of honest signaling, MUP expression is upregulated when males perceive a challenger (Garratt et al., 2012) and depressed with senescence (Garratt et al., 2011), and female mice prefer male scent marks with higher MUP concentration (Nelson et al., 2013). Darcin may also signal dominance status because it is a potent attractant of females (Roberts et al., 2012) and may elicit male-male aggression in certain contexts (Chamero et al., 2007). Although differences in MUPs (and other pheromones) between aggressive and nonaggressive males have been identified after fighting (Janotova & Stopka, 2011, Garratt et al., 2012, Harvey et al., 1989), less is known about variation in pheromone levels within individuals directly competing in the presence of reproductive resources. Moreover, aggression is often not correlated with dominance status (Wang et al., 2014).

Here, we investigate the association of MUP expression level and the formation of social dominance networks before, during and after social competition. Dominant males might express high levels of MUPs to attract females to their territory or to send a warning signal to nondominants; similarly, nondominants might express low levels to avoid attacks from dominants. We therefore predicted that the expression level of darcin and non-darcin MUPs could be used as an honest indicator of social dominance status, and that high expression prior to competition would be predictive of competitive outcomes. Although our experiment was not designed to determine whether honesty of MUP expression is due to handicap or index mechanisms, we were able to address some predictions made by these models (Davies et al., 2012). The handicap model predicts signal intensity is variable within males and that high-quality individuals are favored to produce stronger signals because they can afford the cost; in the absence of competition, investment in signaling may decrease because there is little to be gained. Conversely, the index hypothesis predicts signal intensity varies less within males and is determined by a physical or physiological trait underlying dominance.

Based on observations that MUP expression is plastic (Garratt et al., 2012, Garratt et al., 2011, Janotova & Stopka, 2011, Nelson et al., 2013), we predicted that dominant males would respond to social competition with a greater increase in MUP expression than nondominants, and that this change would be unaffected by body mass, the most common constraint hypothesized to underlie index signals. In addition, because dominance status is heritable in mice, we predicted that MUP expression level before, during and after competition might be affected by paternal dominance status.

Materials and methods

Mice and social competition

See Cunningham et al. 2013 for complete methods of social competition experiments, which are briefly outlined here. Outbred, wild-derived mice (*Mus musculus*) maintained for 13 generations were used in this study. Mice were 44 weeks old, which is within the young-adult life stage for wild-derived mice (Garratt et al., 2011) and an acceptable age for behavioral analysis (Garratt et al., 2012, Ruff et al., 2015). All protocols were approved by the University of Utah Animal Care and Use Committee.

Seminatural enclosures (140 x 60 x 15cm) were composed of a small opaque preferred territory connected to a large transparent suboptimal territory. The preferred site (15 x 30 x 15cm) contained food, water, and nesting material. The suboptimal site had food and water and no nesting material, though provided multiple small retreats. A remotely operated door allowed physical separation of the preferred and communal areas before researchers entered the testing room. A virgin, unrelated, randomly selected female mouse that was singly housed for two weeks was added to each enclosure to further motivate competition. The two-week isolation standardized recent female social experience and reduced possible behavioral effects due to previous housing with a variable number of female littermates. Females were found in the preferred nesting site an average of 71% of the observations, suggesting the male who controlled the preferred site could also partially limit access to the female. One week before competition began, passive integrated transponders were implanted between the scapulae of males for rapid identification and to reduce disturbance during testing.

Mice were grouped into 13 competition arenas (n = 52 males). Males were randomly grouped after selection based on the dominance status of their sire. Sire dominance status was determined using the protocol described here, except sires were assessed using two consecutive rounds of competition rather than one (Cunningham et al., 2013). Sires were removed from breeding cages before parturition and did not interact with their progeny. In this study, each arena consisted of one son from a two-time winning sire, two sons from different one-time winning sires, and one son from a zero-winning sire. Before trials, mice were inspected for abnormalities, weighed, and ear punched. Order of placement into an arena and ear punches were assigned randomly and had no influence on dominance status. Mice were weighed again upon removal from arenas. Mice that appeared under duress were removed from the experiment. After competition, males were individually housed.

To determine dominance status, we took three standard measurements of dominance (Benton et al., 1980, Kuse & De Fries, 1976) previously validated for this population (Cunningham et al., 2013). First, each day a morning, afternoon, and night observation was made to identify the male holder of the preferred nesting site. Observation times were based on daily resting periods for this population. Second, males were assessed for quantities of conspicuous superficial bite marks on the tail and back separately. Third, we measured body mass before, during and after competition. Dominant males were classified by occupying the nest site, a lack of superficial wounding, and retention of body mass throughout the study.

Urinary MUP expression, creatinine and SDS-PAGE

Urinary MUPs and creatinine (a metabolite of muscular creatinine and an index of hydration) were assessed four weeks before competition when males were individually housed (“before”), upon removal from arenas and placement back into individual cages (“during”), and after three-weeks of individual housing following the competition (“after”). We obtained urine samples from 50 males before competition, 29 during competition, and 32 after competition, and recorded 111 urinary protein measurements and 108 creatinine measurements. Although a 24-hour collection regime would have allowed ideal partitioning of the observed variance to either individual or random error (and therefore increased power to detect differences between groups (Blainey et al., 2014)), wild-derived mice are highly sensitive to handling. We therefore chose to use a conservative “snap-shot” sampling approach by systematically collecting urine at standardized times of day while being blind to dominance status; collection was only attempted twice at each timepoint (over a 24 hour period). We address this incomplete sampling with statistical methods that do not produce biased parameter estimates (see Statistical analyses below). Urine was sampled by ‘scruffing’ (grasping the loose skin around the neck and scapulae region) mice over an acrylic sheet, collected by micropipettor, and stored in eppendorf tubes placed on dry ice until moved into a -70°C freezer. Following a 1:8 dilution of whole urine, total urinary protein concentration was determined with the colorimetric Bradford assay (Pierce) using a 96-well plate spectrophotometer (Bio-Tek Synergy HT) according to manufacturer’s instructions. Creatinine in undiluted urine was measured with Jaffe’s basic picrate method (Stanbio Liquicolor Kit), according to the manufacturer’s instructions, and analyzed on 96-well plates using a spectrophotometer as above.

A subsample of the total population was also analyzed with SDS-PAGE gels across the three timepoints. PAGE-analysis confirmed there were no other proteins at high abundance in the male’s urine other than MUPs. Additionally, the MUP isoform darcin is resolvable by PAGE analysis and we tested whether this female attractant was associated with social competition and dominance status. Diluted urine samples were resolved on Any kD Bio-Rad SDS-PAGE gels as described (Roberts et al., 2010). We ran 25 before competition samples, 22 during competition samples, and 19 after competition samples on gels organized by timepoint with roughly equal numbers of dominants and nondominants. We biased our samples towards males that had as complete urine series as possible. To directly assess changes in within-individual MUP expression while controlling for between gel variation, a random subset of individuals ($N = 7$ dominant and nondominant each) at the before and during competition timepoints were ran in tandem on single gels. Urine samples were

diluted 1:90 (wild mice) or 1:60 (C57BL/6), mixed 1:1 with sample buffer (100 mM DTT), heated for 5 minutes at 95° C, then cooled at room temperature for 5 minutes. Gels were run at a constant 200 V and stained with colloidal blue (Novex), and confirmed that > 95% of urinary proteins were MUPs. Expression level of the putative MUP band (~19–21 kDa; Armstrong et al., 2005) and darcin band (~17 kDa) was assessed by measuring their densities within a rectangle of standardized dimensions applied to every gel lane using ImageJ (NIH). To determine the relative portion of total MUP output attributable to darcin, we quantified the darcin index as (darcin density/[darcin + MUP density]). MUP and darcin band densities were normalized to those of a C57BL/6 male urine sample run on every gel. The individual-specific normalized densities of the MUP band, darcin band, and darcin index were highly repeatable across gels (MUP band: $R^2 = 0.70$, $P < 0.0001$; darcin band $R^2 = 0.77$, $P < 0.0001$; darcin index $R^2 = 0.85$, $P < 0.0001$).

In-gel digestion and mass spectrometry

To confirm the identity of the darcin band, three 17 kDa bands (including one from the C57BL/6 urine standard) were excised and de-stained twice in 50% methanol with 50 mM ammonium bicarbonate (NH_4HCO_3) while being gently vortexed for one hour. Gel slices were re-hydrated in 1 ml of 50 mM NH_4HCO_3 for 30 min, cut into pieces, and dehydrated in 1 ml of 100% acetonitrile for 30 min with gentle shaking. Acetonitrile was carefully removed prior to proteolytic digestion. Ten to 20 μL of sequence-grade modified trypsin (Promega) (20 ng/ μL) in 50 mM NH_4HCO_3 was absorbed into gel pieces and incubated overnight at 37°C. Digestion was quenched with 1% formic acid, and dissolved peptides were extracted. Further extraction from the gel material was performed twice by the addition of 50% acetonitrile with 1% formic acid and sonicated at 37°C for 20 minutes. Final dehydration of the gel pieces was accomplished by addition of 100% acetonitrile and incubation at 37°C for 20 minutes. Combined extracted peptides were dried in a vacuum centrifuge (Speed-Vac) and reconstituted in 5% acetonitrile with 0.1% formic acid for LC-MS/MS analysis.

Peptides were analyzed using a nano-LC-MS/MS system comprised of a nano-LC pump (Eksigent) and a LTQ-FT mass spectrometer (ThermoElectron Corporation, San Jose, CA). The LTQ-FT is a hybrid mass spectrometer with a linear ion trap and a Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometer, and equipped with a nanospray ion source (ThermoElectron Corp.). Five to 20 fmoles of tryptic digest were dissolved in 5% acetonitrile with 0.1% formic acid and injected onto a C18 nanobore LC column for nano-LC-MS/MS. A linear gradient LC profile was used to separate and elute peptides, consisting of 5 to 70% solvent in 78 minutes with a flow rate of 350 nL/minute. LTQ-FT MS was operated in data-dependent acquisition mode (Xcalibur 1.4 software) where the most intense peaks in an FT primary scan are determined on-the-fly and trapped for MS/MS analysis and peptide fragmentation in the LTQ linear ion trap portion of the instrument. Spectra in the FT-ICR were acquired from m/z 400 to 1700 at 50,000 resolving power with about 3 ppm mass accuracy. LTQ linear ion trap parameters were as follows. Precursor activation time: 30 ms; activation Q : 0.25; collision energy: 35%; dynamic exclusion width: 0.1 Da to 2.1 Da with one repeat count and duration of 10 s.

LTQ-FT MS data files were processed to peak lists with BioworksBrowser 3.2 (ThermoElectron Corp., San Jose, CA). Parameters used to generate peak lists were as follows: precursor mass: 401-5500 Da; grouping allowing 5 intermediate MS/MS scans; precursor mass tolerance: 5 ppm; minimum ion count in MS/MS: 15; minimum group count: 1. With MASCOT search engine (Matrix Science Ltd) peptide fragments were searched against a custom database comprising all MUP sequences from the Ensemble genome browser (i.e., MUP 1 to MUP 21). Identified peptides were accepted only when the MASCOT ion score value exceeded 20. Four peptides > 15 amino acids long that are unique to darcin were identified in each sample analyzed: VFVEYIHVLENSLALK; AGEYSVTYDGSNTFTILK; FAQLSEEHGIVRENIIDLTNANR; DGETFQLMELYGREPDLSDDIKEK.

Statistical analyses

Effects of competition for dominance status on MUP and darcin expression—

To test for associations between MUPs expression and experimental variables we used linear mixed models (LMMs) with total urinary MUPs (as measured by the Bradford colorimetric assay) as the dependent variable. Before analysis, all data were checked for normality using a Shapiro-Wilks test. Four factors representing *a priori* hypotheses were always included in the LMMs: dominance status, creatinine, timepoint, and a dominance status x time interaction. To account for repeated measures, we included male ID as a random factor. Birthcage was added as a random effect to control for effects of common heritage (either, genetic or environmental). Because dominant and nondominant are terms relative to specific groups, enclosure was modeled as a random blocking effect with male ID nested within enclosure. Other factors that might influence our data, such as, body mass and creatinine x time interaction, were originally included in our models but dropped if non-significant. Body mass did not explain a significant amount of variation in MUP expression in any of the models we analyzed. LMMs handle incomplete datasets better than other statistical methods (Rubin, 1976), and this approach allowed us to explain as much of the variance in the data with as few factors as possible.

To further dissect overall effects from the repeated measures model, we next tested *a priori* contrasts between dominants and nondominants at each timepoint for total urinary MUPs. Fixed and random effects were otherwise identical to the repeated measures model described above, where applicable. The before-competition contrast tested the prediction that MUP expression would be predictive of dominance status. The during-competition contrast tested the prediction that MUP expression would be responsive towards dominance status. The after-competition contrast assessed how MUP expression responded to prior experience of social competition. These contrasts were also applied to the SDS-PAGE density data. The MUP band density, darcin band density, and darcin index were analyzed separately. Correlating densities with the established Bradford colorimetric assays using a Pearson correlation test validated the SDS-PAGE data.

Next, we assessed whether higher MUP expression in dominants relative to nondominants was due to greater upregulation or consistently higher expression. For the subset of males for which we had measurements at both timepoints, we preformed paired T-tests with

creatinine-residual total urinary MUPs (Bradford data), MUP band density, darcin band density, and darcin index separately for the before-to-during samples. We then performed across-group comparisons with these same data between dominants and nondominants.

Effect of sire dominance status on MUP expression—We first used a repeated measures LMM to isolate the effects of sire dominance status on total urinary MUP expression in competing sons with the Bradford colorimetric data. A son's creatinine, dominance status, timepoint, and the sire's dominance status from both the first and second round of competition (along with the first/second round interaction) were modeled as fixed effects. Male ID was nested within enclosure and modeled as a random blocking effect. Next, we used LMMs for *a priori* contrasts at each timepoint modeled with the same factors as the repeated measures model, if applicable.

Effects of competition for dominance status on hydration—We conducted three tests to identify possible effects of social competition and dominance status on hydration as measured by creatinine. First, to determine covariation between creatinine and MUP expression in all males, we used a Pearson correlation test at each timepoint. Second, to determine whether MUP/creatinine slopes were different across timepoints we used an LMM, with urinary MUPs as the dependent variable and timepoint, creatinine, and timepoint x creatinine as covariates; birthcage was included as a random blocking effect. Third, to test whether dominant and nondominants had different creatinine levels, we used repeated measures LMM with creatinine as the dependent variable, but otherwise variables were identical to the repeated measures Bradford colorimetric model. To determine covariation between creatinine and body mass, we used a linear regression across all timepoints. All statistics were executed with JMP (v9.0.1).

Results

Urinary MUPs increase during social competition and are higher in dominant males

Repeated measures LMM analysis (summarized in Table S1) revealed that total urinary MUP expression, as measured by the Bradford assay, increased by 14% on average over the course of the three timepoints (before-to-during, $t_{78} = 2.98$, $P = 0.004$; during-to-after, $t_{60} = 1.33$, $P = 0.189$; Fig. 1a). Total urinary MUP expression was also associated with dominance status ($F_{1,107} = 4.51$, $P = 0.037$; Fig. 1b), timepoint ($F_{2,107} = 9.33$, $P = 0.0003$), and creatinine ($F_{1,107} = 34.07$, $P < 0.0001$), but not the dominance status x timepoint interaction ($F_{2,107} = 1.93$, $P = 0.15$). Analysis of hydration (creatinine model summarized in Table S2) revealed an effect of timepoint ($F_{2,105} = 8.37$, $P = 0.0005$; Fig. 1c), with no difference for the before-to-during comparison ($t_{75} = 0.99$, $P = 0.324$), but increased hydration (i.e., lower creatinine) for the after timepoint (during-to-after $t_{57} = -3.81$, $P = 0.0003$; Fig. 1c).

The average MUP concentration across our study was 3.85 ± 0.933 (mean $\pm$ SE) mg/ml, a low level compared to studies of wild-derived mice (Stopka et al., 2007), and closer that of laboratory (both inbred (Cheetham et al., 2009) and outbred (Sharro et al., 2002)) strains. These low values may be result of over ten generations of breeding in captivity, which might be a selective pressure for reduced MUP expression (Nelson et al., 2013, Sampsell & Held, 1985, Cheetham et al., 2009).

Urinary MUP expression level correlates with dominance during competition

Complete results of our *a priori* contrasts between dominants and nondominants are summarized in Table S3. Before competition, total MUP expression as measured by the Bradford assay was associated with creatinine ($F_{1,48} = 6.67$, $t_{48} = 2.58$, $P = 0.013$), but not dominance status ($F_{1,48} = 3.24$, $t_{49} = -1.80$, $P = 0.079$; Fig. 1d). During competition, dominant males had 24% greater MUP expression than nondominants, and expression was associated with dominance status ($F_{1,28} = 5.46$, $t_{28} = -2.34$, $P = 0.029$; Fig. 1d) and creatinine ($F_{1,28} = 14.86$, $t_{28} = 3.85$, $P = 0.0009$). After competition, MUP expression was not associated with creatinine ($F_{1,31} = 0.72$, $t_{31} = 0.85$, $P = 0.403$) or dominance status ($F_{1,31} = 0.12$, $t_{31} = 0.85$, $P = 0.732$; Fig. 1d).

Dominant males have higher darcin expression before and during competition

Densitometry values of MUP expression (as measured by the sum of normalized MUP-band density + darcin-band density) were positively correlated with Bradford colorimetric values ($R^2 = 0.55$, $P < 0.0001$; Fig. 2a, b), validating densitometry as an accurate measurement of MUP expression.

We next used the three *a priori* LMM contrasts for analyzing normalized density data (results summarized in Table 1). MUP densities were first derived from gels where dominant and nondominants were grouped according to timepoint (Fig. 2a). Before competition, dominant males did not have higher MUP-band density (Fig. 2c), but did have higher darcin-band density (Fig. 2d) and darcin index (darcin density/[darcin + MUP] density; Fig. 2e). During competition, dominant males had higher darcin-band density (Fig. 2d), MUP-band density (Fig. 2c), and darcin index (Fig. 2e) than nondominant males. None of the three measures were different between dominants and nondominants after competition. These results were confirmed when MUP densities were derived from gels where the same individuals were run in tandem for the before and during competition timepoints on the same gel (Fig. S1).

Among all individuals, paired T-tests of residual MUPs (correcting for hydration as measured by creatinine) revealed significant upregulation of urinary MUPs ($t_{27} = 2.32$, $P = 0.021$; Fig. 3a) and MUP-band density ($t_{16} = 3.26$, $P = 0.004$; Fig. 3b) from the before-to-during competition timepoint. There was no change in darcin-band density ($t_{16} = 0.71$, $P = 0.484$; Fig. 3c) or darcin index ($t_{16} = 0.17$, $P = 0.865$; Fig. 3d). Across-groups paired comparisons between dominant and nondominant males showed no differences in the slopes of urinary MUPs as measured by the Bradford assay ($F_{1,27} = 0.02$, $P = 0.88$; Fig. 3a), MUP-band density ($F_{1,16} = 0.18$, $P = 0.676$; Fig. 3b), darcin-band density ($F_{1,16} = 0.15$, $P = 0.698$; Fig. 3c), or darcin index ($F_{1,16} = 0.93$, $P = 0.349$; Fig. 3d) from the before-to-during competition timepoint. Comparison of means between this subset of individuals was consistent with LMM results and showed that dominant males had higher expression of urinary MUPs ($F_{1,27} = 8.91$, $P = 0.006$) and higher density of the darcin band ($F_{1,16} = 8.12$, $P = 0.012$), but not higher density of the MUP band ($F_{1,16} = 1.29$, $P = 0.272$). Equivalent results were obtained when between-timepoint samples per individual were run on the same or different gels. Thus, dominant males have higher levels of MUPs and darcin, but they do not upregulate at a greater rate than nondominants during competition.

Sire dominance status modulates MUP expression in sons

LMM analysis of paternal effects (complete results summarized in Table S4) showed that the sire winning his second round of competition unexpectedly had a negative effect on total MUP expression in his sons ($t_{107} = 2.30$, $P = 0.026$); this model confirmed effects of creatinine ($F_{1,107} = 35.52$, $P < 0.0001$), dominance status ($F_{1,107} = 6.60$, $P = 0.014$) and timepoint ($F_{1,107} = 18.62$, $P < 0.0001$). LMM contrasts at each timepoint separately revealed this effect was manifest before competition ($t_{48} = 2.57$, $P = 0.014$), but not significant during ($t_{27} = 0.22$, $P = 0.831$) or after ($t_{31} = 0.41$, $P = 0.688$; Table S5). These contrasts confirmed effects of dominance and creatinine at the before and during, but not after competition timepoints (Table S5). Post hoc analysis showed that although sons of winner fathers had downregulated MUP expression before competition, they had significantly upregulated expression during and after competition (Fig. 4). Consistent with our much larger analysis of the heritability of dominance status, sires that won the second round were more likely to have dominant sons (11/31 sons were dominant) than sires who lost the second round (2/21 sons were dominant (Fisher's exact test: $P = 0.053$, odds ratio = 0.19, 95% CI: 0.03 – 0.98; see also (Cunningham *et al.*, 2013)).

Hydration varies with social context and is not stably correlated with body mass or MUPs

The average urinary creatinine level in male mice in our study was 0.0780 ± 0.033 (mean $\pm$ SE) mg/ml, in agreement with previous reports (Dunn *et al.*, 2004). The correlation between total MUP expression and creatinine was marginally significant before competition ($R^2 = 0.0703$, $P = 0.06$), significant during competition ($R^2 = 0.383$, $P = 0.0006$), and non-significant after competition ($R^2 = 0.0126$, $P = 0.54$; Fig. S2). LMM analysis of the relationship between MUP expression and creatinine showed these slopes were significantly different (creatinine x time interaction: $F_{2, 105} = 3.444$, $P = 0.036$); this analysis also confirmed an effect of creatinine ($F_{1, 106} = 4.505$, $P = 0.036$) and timepoint ($F_{2, 104} = 6.966$, $P = 0.002$) on MUP expression. Finally, our analysis showed no relationship between body mass and creatinine across all timepoints (Fig. S3). Thus, creatinine does not have an isometric relationship with urinary MUP expression here, but does explain a significant amount of variation in MUP expression when used as a covariate in a linear model.

Discussion

A central question in signaling theory is what keeps signals honest over evolutionary time (Davies *et al.*, 2012). In house mice, urinary MUPs are excreted in extraordinarily large, potentially costly amounts, and it has been hypothesized that MUP expression level is an honest signal of quality in males (Garratt *et al.*, 2012, Garratt *et al.*, 2011, Gosling *et al.*, 2000, Stopkova *et al.*, 2007, Janotova & Stopka, 2011, Beynon & Hurst, 2004). Consistent with the honest signal hypothesis, we show that dominant males have higher expression levels of total MUPs than nondominants during competition and that darcin expression levels are higher in dominants during and prior to competition, making it predictive of dominance status. We were also able to test some opposing predictions of the handicap and index models of honest signaling in regards to intra- and inter-individual variation (Davies *et al.*, 2012). Consistent with the handicap model, we show that non-darcin MUP expression level is upregulated during social competition across all competitors and is highest in

dominant males, suggesting MUP expression may be costly and therefore down-regulated in the absence of, or at least prior to, social competition. A common prediction of the index model is that signal intensity will be determined by body size, although we found no discernable quantitative effects of body mass on MUP expression level. However, the index hypothesis also predicts that signal intensity should vary less within individuals because it is mechanistically impossible to cheat, and our intra-individual analysis revealed that darcin expression was stable, but higher in dominants, from before to during competition. This latter result suggests that an underlying physiological trait might govern darcin expression level and cannot be faked.

Urinary darcin is a known pheromone (Chamero et al., 2007, Roberts et al., 2012, Roberts et al., 2010), and we evaluated this MUP isoform over time using SDS-PAGE densitometry. Darcin expression was higher both before and during competition in dominant males, making it a predictive molecular marker for dominance status. Our results raise the possibility that because dominant males excrete higher levels of these proteins they attract more mating opportunities with females (Roberts et al., 2012, Nelson et al., 2013), or send an honest warning signal to potential male competitors (Chamero et al., 2007). Similarly, nondominant males might have low expression levels to avoid agonistic encounters.

Intriguingly, dominance status in sires was negatively correlated with MUP expression in socially naïve sons with whom the sire never had direct contact. This apparent repression of MUP expression was released once competition ensued, when the sons of dominant males significantly upregulated MUP expression. A study on a wild-derived mouse line related to the mice in this study showed that intense social competition among sires negatively regulates MUPs in sons, while maternal experience of social competition upregulates MUPs in sons (Nelson et al., 2013). Taken together, these paternal effects on MUP expression are suggestive of a possible parent-offspring conflict. When male mice breed in a defended territory, maturing sons become competitors and will often supplant their father (Gerlach, 1996). Dominant males might therefore maximize lifetime reproductive success (and mitigate territorial threats) by transiently decreasing MUP expression in pre-dispersal sons. Similarly, sons who will be dominant later in life might signal subordinate status relative to fathers to decrease agonistic encounters until dispersal from the natal territory. This association between sire dominance status and pheromone expression in sons identifies a potential mechanism maintaining group stability that could be mediated through several different non-mutually exclusive mechanisms, such as direct and indirect genetic effects, epigenetic inheritance, or maternal effects.

How hydration (as measured by creatinine) responds to social competition is less understood than MUP expression and competition. The effect of hydration on MUP concentration is generally standardized with a MUP/creatinine ratio (Stopkova et al., 2007, Cheetham et al., 2007, Beynon & Hurst, 2004). However, our analysis found that the association between MUPs and creatinine was not stable across the entire study. Creatinine explained statistically significant amounts of variation in MUPs only at the before and during timepoints. We also identified a conspicuous drop in creatinine levels after competition, which is possibly due to unrestricted access to water after a stressful experience of social competition. MUPs and creatinine are therefore not stably related—a necessary condition for a ratio to properly

control for variation (e.g., Allison et al., 1995). Adding creatinine as a covariate to a statistical model addresses this limitation (Packard & Boardman, 1988, Allison et al., 1995, Wagner et al., 2010).

Our results correlate MUPs in general and darcin in particular with dominance status, but they do not demonstrate a causal relationship. To better understand whether MUP expression level has evolved to be an honest indicator, and that other mice use this signal, more questions need to be addressed. First, what is the causal effect of MUP or darcin expression level on dominance status? Experiments manipulating MUP or darcin expression levels through molecular genetics means (e.g., recombinant viral vectors) and assessing social dominance status will help address this question. Second, what is the social role of the volatile chemical compounds that MUP/darcin carry and release (e.g., SBT)? Experimental biochemical manipulations of volatiles, MUPs with volatiles, and MUPs without volatiles during the formation of social dominance networks may provide insight into this question. Finally, more work is needed to determine the extent to which MUPs are condition-dependent handicap signals, or indexes of dominance status. Experiments combining quantitative genetics of social dominance and physiological manipulations (e.g., stress) may shed light on the degree to which MUP expression is constrained by genetic inheritance or current physiological state.

MUPs are expressed in extraordinarily high and potentially costly quantities in urine (Beynon & Hurst 2004), a trait that has captured the attention of biologists for decades (Finlayson et al., 1965). This unique trait is illustrated by the notion that a 25 g mouse that excretes 2 ml of urine per day at a MUP concentration of 25 mg/ml will lose 50 mg of protein per day (or 0.2% of total body weight per day). That level of investment exceeds the investment of a peacock in its iconic train by approximately 12 fold per year (as measured by weight and corrected for body weight; (Petrie et al., 1996)). By showing that dominant males express more urinary MUPs than nondominants, and that increased expression of the MUP isoform darcin is predictive of dominance status, this work identifies a potential source of selection on increasingly higher expression levels of MUPs in house mice.

Social signals are central to the regulation of many animal societies. Social information is processed through many sensory modalities, and olfaction seems to be of particular importance (Wyatt, 2014). Our results illustrate both the predictive and dynamic nature that pheromones can have in relation to the formation of a social network. Our result that highly competitive sires depress their son's pheromone levels, even without direct physical interaction, suggests that the regulation of social signals is quite complex. Our results motivate further tests of the costs and constraints of signal production. Understanding the relationship between the signals produced by an individual and their relationship to social networks is the first step in elucidating the evolutionary forces shaping social signals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Fred Adler, David Carrier, Elizabeth Cashdan, Terry Dial, Allen Moore, Nadja Schilling, and Jon Seger for insightful discussions. Mathew Mulvey kindly access to provided a photospectrometer. We thank Catherine Dulac for support. This work was funded through the University of Utah Funding Incentive Seed Grant Program to David R. Carrier. Additional funding was provided by NSF grants to David R. Carrier (IOS 08-17782) and WK Potts (DEB 09-18969), an NIH grant to WK Potts (RO1-GM109500). CB Cunningham and JS Ruff were supported by NSF DGE 08-41233 Educational Outreach fellowships. AC Nelson was supported by grants NIH T32 and NSF DIGG 09-14244.

References

- Allison DB, Paultre F, Goran MI, Poehlman ET, Heymsfield SB. Statistical considerations regarding the use of ratios to adjust data. *International Journal of Obesity and Related Metabolic Disorders*. 1995; 19:644–652. [PubMed: 8574275]
- Armstrong SD, Robertson DH, Cheetham SA, Hurst JL, Beynon RJ. Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. *Biochem J*. 2005; 391:343–50. [PubMed: 15934926]
- Benton D, Dalrymple-Alford J, Brain F. Comparisons of measures of dominance in the laboratory mouse. *Animal Behaviour*. 1980; 28:1274–1279.
- Beynon RJ, Hurst JL. Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*. *Biochem Soc Trans*. 2003; 31:142–6. [PubMed: 12546672]
- Beynon RJ, Hurst JL. Urinary proteins and the modulation of chemical scents in mice and rats. *Peptides*. 2004; 25:1553–63. [PubMed: 15374657]
- Blainey P, Krzywinski M, Altman N. Points of Significance: Replication. *Nat Meth*. 2014; 11:879–880.
- Bronson F. The reproductive ecology of the house mouse. *The Quarterly Review of Biology*. 1979; 54:265–299. [PubMed: 390600]
- Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, Cravatt BF, Stowers L. Identification of protein pheromones that promote aggressive behaviour. *Nature*. 2007; 450:899–902. [PubMed: 18064011]
- Cheetham SA, Smith AL, Armstrong SD, Beynon RJ, Hurst JL. Limited variation in the major urinary proteins of laboratory mice. *Physiol Behav*. 2009; 96:253–61. [PubMed: 18973768]
- Cheetham SA, Thom MD, Jury F, Ollier WE, Beynon RJ, Hurst JL. The genetic basis of individual-recognition signals in the mouse. *Current Biology*. 2007; 17:1771–7. [PubMed: 17949982]
- Clutton-Brock TH, Albon SD. The Roaring of Red Deer and the Evolution of Honest Advertisement. *Behaviour*. 1979; 69:145–170.
- Cunningham C, Ruff J, Chase K, Potts W, Carrier D. Competitive Ability in Male House Mice (*Mus musculus*): Genetic Influences. *Behavior Genetics*. 2013; 43:151–160. [PubMed: 23291957]
- Davies, NB.; Krebs, JR.; West, SA. *An Introduction to Behavioural Ecology*. 4. Wiley-Blackwell; Oxford, UK: 2012.
- DeFries JC, McClearn GE. Social dominance and Darwinian fitness in the laboratory mouse. *Am Nat*. 1970; 104:408–411.
- Desjardins C, Maruniak JA, Bronson FH. Social rank in house mice: Differentiation revealed by ultraviolet visualization of urine marking patterns. *Science*. 1973; 182:939–941. [PubMed: 4745598]
- Dewsbury DA. Dominance Rank, Copulatory Behavior, and Differential Reproduction. *The Quarterly Review of Biology*. 1982; 57:135–159. [PubMed: 7051088]
- Diep SK, Westneat DF. The integration of function and ontogeny in the evolution of status signals. *Behaviour*. 2013; 150:1015–1044.
- Drickamer LC. Urine marking and social dominance in male house mice (*Mus musculus domesticus*). *Behavioural Processes*. 2001; 53:113–120. [PubMed: 11254998]
- Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K. Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int*. 2004; 65:1959–1967. [PubMed: 15086941]

- Ellis L. Dominance and reproductive success among nonhuman animals: A cross-species comparison. *Ethology and Sociobiology*. 1995; 16:257–333.
- Finlayson JS, Asofsky R, Potter M, Runner CC. Major urinary protein complex of normal mice: origin. *Science*. 1965; 149:981–2. [PubMed: 5827345]
- Fox SF, Heger NA, Delay LS. Social cost of tail loss in *Uta stansburiana*: lizard tails as status-signalling badges. *Animal Behaviour*. 1990; 39:549–554.
- Garratt M, McArdle F, Stockley P, Vasilaki A, Beynon RJ, Jackson MJ, Hurst JL. Tissue-dependent changes in oxidative damage with male reproductive effort in house mice. *Functional Ecology*. 2012; 26:423–433.
- Garratt M, Stockley P, Armstrong SD, Beynon RJ, Hurst JL. The scent of senescence: sexual signalling and female preference in house mice. *Journal of Evolutionary Biology*. 2011; 24:2398–2409. [PubMed: 21848973]
- Gerlach G. Emigration mechanisms in feral house mice- a laboratory investigation of the influence of social structure, population density, and aggression. *Behavioral Ecology and Sociobiology*. 1996; 39:159–170.
- Gosling LM, Roberts SC, Thornton EA, Andrew MJ. Life history costs of olfactory status signalling in mice. *Behav Ecol Sociobiol*. 2000; 48:328–332.
- Grafen A. Biological signals as handicaps. *Journal of Theoretical Biology*. 1990; 144:517–546. [PubMed: 2402153]
- Harvey S, Jemiolo B, Novotny M. Pattern of volatile compounds in dominant and subordinate male mouse urine. *J of Chemical Ecology*. 1989; 15:20612072.
- Holekamp, KE.; Dloniak, SM. Maternal effects in fissiped carnivores. In: Mateo, JM.; Maestripieri, D., editors. *Maternal Effects in Mammals*. University of Chicago Press; Chicago: 2009. p. 227-255.
- Holman L. Costs and constraints conspire to produce honest signaling: insights from an ant queen pheromone. *Evolution*. 2012; 66:2094–2105. [PubMed: 22759287]
- Hurst JL, Robertson DHL, Tolladay U, Beynon RJ. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim Behav*. 1998; 55:1289–1297. [PubMed: 9632512]
- Janotova K, Stopka P. The Level of Major Urinary Proteins is Socially Regulated in Wild Mus musculus musculus. *Journal of Chemical Ecology*. 2011; 37:647–656. [PubMed: 21594616]
- Jemiolo B, Alberts J, Sochinski-Wiggins S, Harvey S, Novotny M. Behavioral and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. *Animal Behaviour*. 1985; 33:1114–1118.
- Kaur AW, Ackels T, Kuo TH, Cichy A, Dey S, Hays C, Kateri M, Logan DW, Marton TF, Spehr M, Stowers L. Murine pheromone proteins constitute a context-dependent combinatorial code governing multiple social behaviors. *Cell*. 2014; 157:676–88. [PubMed: 24766811]
- Koyama S, Soini HA, Foley JG, Novotny MV, Lai C. Cell proliferation in the subventricular zone by synthetic murine pheromones. *Frontiers in Behavioral Neuroscience*. 2013; 7
- Kuse A, De Fries J. Social dominance and Darwinian fitness in laboratory mice: an alternative test. *Behavioral Biology*. 1976; 16:113–116. [PubMed: 943157]
- Maynard Smith J, Harper DGC. Animal Signals: Models and Terminology. *Journal of Theoretical Biology*. 1995; 177:305–311.
- Moore PJ, Reagan-Wallin NL, Haynes KF, Moore AJ. Odour conveys status on cockroaches. *Nature*. 1997; 389:25–25. [PubMed: 9288961]
- Mudge JM, Armstrong SD, McLaren K, Beynon RJ, Hurst JL, Nicholson C, Robertson DH, Wilming LG, Harrow JL. Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. *Genome Biol*. 2008; 9:R91. [PubMed: 18507838]
- Nelson AC, Cauceglia JW, Merkley SD, Youngson NA, Oler AJ, Nelson RJ, Cairns BR, Whitelaw E, Potts WK. Reintroducing domesticated wild mice to sociality induces adaptive transgenerational effects on MUP expression. *Proc Natl Acad Sci U S A*. 2013; 110:19848–53. [PubMed: 24248373]
- Packard GC, Boardman TJ. The Misuse of Ratios, Indices, and Percentages in Ecophysiological Research. *Physiological Zoology*. 1988; 61:1–9.

- Peek FW. An experimental study of the territorial function of vocal and visual display in the male red-winged blackbird (*Agelaius phoeniceus*). *Animal Behaviour*. 1972; 20:112–118.
- Petrie M, Cotgreave P, Stewart I. Variation in the train morphology of peacocks (*Pavo cristatus*). *Journal of Zoology*. 1996; 238:365–371.
- Rich TJ, Hurst JL. Scent marks as reliable signals of the competitive ability of mates. *Anim Behav*. 1998; 56:727–735. [PubMed: 9784223]
- Rich TJ, Hurst JL. The competing countermarks hypothesis: reliable assessment of competitive ability by potential mates. *Animal Behaviour*. 1999; 58:1027–1037. [PubMed: 10564605]
- Roberts SA, Davidson AJ, McLean L, Beynon RJ, Hurst JL. Pheromonal Induction of Spatial Learning in Mice. *Science*. 2012; 338:1462–1465. [PubMed: 23239735]
- Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, Beynon RJ, Hurst JL. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biol*. 2010; 8:75. [PubMed: 20525243]
- Rubin DB. Inference and missing data. *Biometrika*. 1976; 63:581–592.
- Ruff JS, Hugentobler SA, Suchy AK, Sosa MM, Tanner RE, Hite ME, Morrison LC, Gieng SH, Shigenaga MK, Potts WK. Compared to Sucrose, Previous Consumption of Fructose and Glucose Monosaccharides Reduces Survival and Fitness of Female Mice. *The Journal of Nutrition*. 2015
- Saks L, Ots I, Hõrak P. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. *Oecologia*. 2003; 134:301–307. [PubMed: 12647136]
- Sampsel BM, Held WA. Variation in the major urinary protein multigene family in wild-derived mice. *Genetics*. 1985; 109:549–68. [PubMed: 3979814]
- Sharrow SD, Vaughn JL, Židek L, Novotny MV, Stone MJ. Pheromone binding by polymorphic mouse major urinary proteins. *Protein Science*. 2002; 11:2247–2256. [PubMed: 12192080]
- Sherborne AL, Thom MD, Paterson S, Jury F, Ollier WE, Stockley P, Beynon RJ, Hurst JL. The genetic basis of inbreeding avoidance in house mice. *Current Biology*. 2007; 17:2061–6. [PubMed: 17997307]
- Stopka P, Janotova K, Heyrovsky D. The advertisement role of major urinary proteins in mice. *Physiology & Behavior*. 2007; 91:667–670. [PubMed: 17477943]
- Stopkova R, Stopka P, Janotova K, Jedelsky PL. Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). *J Chem Ecol*. 2007; 33:861–9. [PubMed: 17333372]
- Wagner BD, Accurso FJ, Laguna TA. The applicability of urinary creatinine as a method of specimen normalization in the cystic fibrosis population. *Journal of Cystic Fibrosis*. 2010; 9:212–216. [PubMed: 20227353]
- Wang F, Kessels HW, Hu H. The mouse that roared: neural mechanisms of social hierarchy. *Trends in Neurosciences*. 2014; 37:674–682. [PubMed: 25160682]
- Wyatt, TD. *Pheromones and Animal Behaviour: Communication by Smell and Taste*. 2. Cambridge University Press; Cambridge, United Kingdom: 2014.
- Zahavi A. Mate selection—A selection for a handicap. *Journal of Theoretical Biology*. 1975; 53:205–214. [PubMed: 1195756]

Fig. 1.

Effects of social competition and competitive ability on urinary major urinary proteins (MUPs) and creatinine. (a – c) Summary of repeated-measures analysis of total urinary MUPs by Bradford assay (see Table S1). (a) Total urinary MUP expression (mg/ml) versus timepoint (before, during, after). MUP output was for all males was higher during and after competition relative to before. (b) Total urinary MUP expression (mg/ml) versus dominance status (Nondominant, Dominant). Dominant males had higher urinary MUP expression across the experiment than nondominant males. (c) Creatinine (mg/ml) versus timepoint (before, during, after). All males had lower creatinine levels after competition than before and during competition. (d – e) Summary of analysis by timepoint (see Table S2 & S3). (d) Total urinary MUP expression (mg/ml) versus timepoint (before, during, after) grouped by dominance status. Dominant males had greater MUP expression during competition than nondominants. (e) Creatinine (mg/ml) versus timepoint (before, during, after) grouped by dominance status. Creatinine did not differ between dominants and nondominants at any timepoint. Raw Bradford colorimetric data presented for visual purposes only. Lines show means $\pm$ SEM. * $P < 0.05$.

Fig. 2.

SDS-PAGE densitometry of darcin and non-darcin MUPs across all three timepoints (before, during, after). (a) Diluted urine samples were run under reducing conditions and stained with Colloidal Blue. Non-darcin MUPs migrate to ~19–21 kDa and darcin migrates to ~17 kDa. An individual C57BL/6 male sample was run on each gel to normalize densities. Dominant (+), nondominant (–). (b) Total urinary MUP expression (mg/ml) versus the sum of the MUP band densities. The sum of MUP-band + darcin-band density is highly correlated with urinary protein Bradford colorimetric values ($P < 0.0001$). (c – e) densitometry analysis by timepoint (see Table 1). (c) Relative non-darcin MUP band density (~19–21 kDa) versus timepoint (before, during, after) grouped by dominance status (Nondominant, Dominant). Relative to nondominant males, dominant males have higher MUP-band density during competition. (d) Relative darcin band density (~17 kDa) versus timepoint (before, during, after) grouped by dominance status (Nondominant, Dominant). Dominants had higher darcin-band density before and during competition compared to nondominants. (e) Relative darcin % or total MUP density versus timepoint (before, during, after) grouped by dominance status (Nondominant, Dominant). Dominant males have higher darcin index before and during competition. Raw data are presented for visual purposes only. Lines show means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

Fig. 3.

Paired comparisons of MUP expression before and during competition. Among-individual means $\pm$ SEM for all males are shown as open circles on left and right margins. Within-individual measurements show dominant (red dotted lines) and nondominant (grey solid lines) males. (a) Total urinary MUP expression versus timepoint (before and during). MUPs were significantly upregulated among all individuals. (b) Relative non-darcin MUP band density versus timepoint (before and during). MUP-band density was significantly upregulated among all individuals. (c) Relative darcin band density versus timepoint (before and during). There were no differences in darcin-band density. (d) Relative darcin % or total MUP density versus timepoint (before and during). The darcin index was not different between these two timepoints among all individuals. There were no within-individual differences in rates of upregulation between dominant and nondominants. * $P < 0.05$.

Fig. 4.

Paternal effect of social dominance status on MUP expression. Graph shows total urinary MUP expression (mg/ml) versus timepoint (before, during, after) grouped by the dominance status of a competitor's sire in the sire's second round of competition: dominant (Dom) or nondominant (ND). Sires who were dominant their second (and final) round of competition produced sons with downregulated MUP expression before the sons entered competition; their sons then had significantly greater MUP expression during and after competition. Dominant fathers were more likely to produce dominant sons (red dots) than nondominant sons (gray dots). Blue bars show Bradford colorimetric means $\pm$ SEM. Significance values are Tukey HSD. ** $P < 0.01$. *** $P < 0.001$.

LMM analysis of dominance status and expression of MUPs and darcin using gel densitometry. Each timepoint analyzed separately: Before (1.1), during (1.2) and after (1.3) competition. Darcin index is defined by $(\text{darcin-band density} / [\text{darcin-band density} + \text{MUP-band density}])$.

	Darcin-band density						MUP-band density						Darcin index					
	R^2	Estimate	Std Error	t Ratio	P value	R^2	Estimate	Std Error	t Ratio	P value	R^2	Estimate	Std Error	t Ratio	P value			
1.1. Before competition																		
Intercept	0.86	0.08	0.143	0.60	0.55	0.85	0.396	0.217	1.83	0.083	0.87	0.206	0.046	4.47	0.0002			
Dominance status (ND)		-0.110	0.044	-2.47	0.026		-0.061	0.068	-0.90	0.381		-0.032	0.014	-2.27	0.039			
Creatinine		3.90	1.38	2.81	0.015		7.03	2.12	3.31	0.006		0.631	0.439	1.44	0.176			
1.2. During competition																		
Intercept	0.93	0.347	0.129	2.68	0.019	0.93	1.05	0.221	4.78	0.0003	0.93	0.186	0.035	5.18	0.0001			
Dominance status (ND)		-0.223	0.049	-4.55	0.002		-0.299	0.081	-3.68	0.006		-0.067	0.013	-4.92	0.0004			
Creatinine		1.62	1.15	1.41	0.202		3.00	1.89	1.58	0.156		0.626	0.328	1.90	0.088			
1.3. After competition																		
Intercept	0.65	0.274	0.137	2.00	0.065	0.88	0.697	0.236	2.95	0.010	0.62	0.230	0.048	4.75	0.0003			
Dominance status (ND)		0.006	1.84	0.12	0.907		0.036	0.090	0.410	0.692		0.002	0.019	0.13	0.900			
Creatinine		1.59	1.84	0.86	0.404		5.81	2.97	1.96	0.077		0.156	0.653	0.24	0.814			

J Evol Biol. Author manuscript; available in PMC 2016 June 01.