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Foxn1 Gene Knockout Suppresses Sexual Attractiveness and Pheromonal Components of Male Urine in Inbred Mice

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Abstract

The immunocompetence handicap hypothesis (ICHH) posits that females prefer signals emitted by immunocompetent males over immunocompromised males and that these signals are honest. However, mechanisms of mate choice under an ICHH model may be impacted by levels of genetic variation (inbred animals vs. outbred animals). Here, we conducted 2-choice female preference experiments and chemical analyses of male urine in inbred BALB/c and outbred CD-1 mice, both of which have immunocompromised nude (*nu*) strains resulting from a *Foxn1* gene knockout. We found that inbred BALB/c females but not outbred CD-1 females preferred the urine of healthy males over that of immunocompromised males despite measured differences in the qualities of their urine. There was a clear increase in female-attracting pheromones (such as farnesenes) in the preputial glands and urine metabolites in healthy BALB/c males but no such difference between CD-1 and CD-1 *nu* males. Therefore, CD-1 male urine failed to provide an honest mate-choice cue for females. Our results suggest that deleterious traits associated with male odor in mice might be jointly affected by the level of inbreeding and immunodeficiency caused by a single-gene knockout.

Key words: *Foxn1*, handicap hypothesis, mate choice, pheromone

Introduction

Since Zahavi (1975) first proposed the handicap hypothesis, studies on mate choice have continued to build on our understanding of the evolution of reliable signaling. For example, Hamilton and Zuk (1982) were the first to empirically demonstrate that birds with brighter colors have less parasites. This and other pioneering work led to the now widely accepted notion that exaggerated male ornaments can function as honest signals, indicating a low parasite load or high resistance to disease (see Clayton 1991; Andersson 1994; Hamilton and Poulin 1997). By preferentially mating with such males, females are less likely to be infected by parasites and more likely to pass on good genes to their offspring from attractive males (Ilmonen et al. 2009). The evolution of such honest signals under selection pressure from parasites and pathogens can be explained by the immunocompetence handicap hypothesis (ICHH; Falstad and Karter 1992). The hypothesis posits that because testosterone is not only responsible for the production of male secondary sexual traits but is also immunosuppressive, the cost of being able to express sexual traits is decreased immune function (Falstad and Karter 1992; Kurtz and Sauer 1999). The ICCH also predicts that males should have an optimum level of testosterone that maximizes trait expression while minimizing immunosuppression (Verhulst et al. 1999; Roberts et al. 2004). Therefore, only males with high genetic quality or heterozygosity can afford to fully display sexual characteristics without suffering large parasite loads or increases in pathogens (Falstad and Karter 1992; Hillgarth et al. 1997; Roberts et al. 2004; Ilmonen et al. 2009). Although the ICHH has attracted much empirical attention, results from many studies have been ambiguous if not contradictory (see review by Roberts et al. 2004).

The ICHH has stimulated several empirical tests in mammals (Zala et al. 2004), birds (Duffy and Ball 2002; Reid

et al. 2005; Alonso-Alvarez et al. 2007), and insects (Rantala, Kortet, et al. 2003; Rantala, Vainikala, and Koret 2004; Simmons et al. 2005; Vainikka et al. 2007). A recent study by Reid et al. (2005) in song sparrows (*Melospiza melodia*) demonstrated that the situation appears to be more complicated than once considered. In their study, Reid et al. (2005) showed that although males' song repertoire size (a secondary sexual trait) increased with cell-mediated immune response, both repertoire size and cell-mediated immune response decline with levels of inbreeding. Females choosing males with a larger repertoire size on average acquire more outbred and therefore more heterozygous mates than females that mate randomly. This added complexity of inbreeding raises a new challenge for the ICHH—What is the primary reason for females to choose males with exaggerated secondary sexual traits: inbreeding avoidance, immunocompetence, or both? This question remains unanswered for mammals, despite the large number of studies examining ICHH and mammalian systems.

For a number of reasons, mice (*Mus musculus*) are ideal animals to study when addressing questions of immunocompetence and sexual selection. First, mice rely extensively on odor in social interactions and mate choice. For example, the chemical constituents in male odors provide the major, if not only, source of information for females when choosing mates (e.g., Yamazaki et al. 1994; Singer et al. 1997; Penn et al. 1998; Hurst and Rich 1999). Second, it has been shown that parasitic infection reduces immune responsiveness and profoundly affects social rank and aggressiveness in males (Barnard et al. 1998). Third, behavioral assays have confirmed that females can extract information from male odor and discriminate between males infected or uninfected by viruses, bacteria or parasites (Kavaliers and Colwell 1995; Penn et al. 1998; Penn and Potts 1998a, 1998b; Zala et al. 2004; Kavaliers et al. 2005). Recently, Riviére et al. (2009) reported novel vomeronasal organ chemosensors, formyl peptide receptor-like proteins showing an olfactory function, associated with the identification of the ligands pertaining to pathogens or of pathogenic states in mice. These factors provide us with a rare opportunity to examine in detail the components of mouse odor that phenotypically code for olfactory information regarding unhealthy states.

Established *Foxn1* gene knockout mouse models are athymic and T-cell deficient, resulting in immunodeficiency. They are genetically different in only the *Foxn1* gene when compared with corresponding background strains (Isaacson and Cattanach 1962; Schorpp et al. 1997; Schlake 2001). In addition, manipulation of thymus-mediated immunity has been confirmed in outbred CFLP mice to cause a reduction in serum testosterone levels and some social behaviors (Barnard et al. 1997a, 1997b). These mice appear to be ideal models for us to examine immunodeficit-related odor components and mate choice. However, mice behavior may differ markedly between inbred and outbred strains, in addition to being affected by environmental, physiological, and hormonal factors (Crabbe et al. 1999; Nevison et al. 1999; Spearow et al. 1999; Champagne et al. 2007). In addition, the behavioral effect of knocking out a gene depends on the existing gene network (Logue et al. 1997; Kelly et al. 1998; Võikar et al. 2001). For example, gene-based mate preference (such as major histocompatibility complex [MHC]-based) in mice can vary and may be modulated by other genetic factors (Jordan and Bruford 1998).

An ideal way to answer complex questions surrounding the IHCC is to use immunodeficient strains (*Foxn1* gene knockout) of outbred (e.g., CD-1 lines of more diverse genetic background) and inbred mice (BALB/c lines of less diverse genetic background). Comparing the responses of females to CD-1 with CD-1 *nu* males and BALB/c verses BALB/c *nu* males provides an opportunity for us to explore the role of genetic factors in modulating social odor while testing the ICHH. Examining how females respond to male odors with different pheromone compositions would give us insights into olfactory decision-making rules used by females in mate choice and on the role of genetic correlates between pheromones and immunity.

Although how the dysfunctional *Foxn1* leads to skin nudity is well understood (Mecklenburg et al. 2001), its potential pleiotropic effect on odor constituents has yet to be systematically explored. Because knocking out *Foxn1* is known to induce profound changes in some sebum hormones in NMRI versus NMRI *nu* mice (Köpf-Maier and Mboneko 1990), we speculated that the expected *Foxn1* knockout-induced alterations of androgen as well as immunocompetence in CD-1 *nu* and BALB/c *nu* mice might change the pheromone composition of mice odor. Under the ICHH, we would expect that the urinary attractiveness and pheromone compounds used by female mice in mate choice for CD-1 *nu* and BALB/c *nu* male mice would be modified due to gene knockout. Fortunately most, if not all, male pheromone compounds that are attractive to females have been systematically identified. They include *E*-β-farnesene, *E*,*E*-α-farnesene, hexadecanol, and hexadecanol acetate, which are secreted into voided urine by the preputial glands (Novotny et al. 1990; Jemiolo et al. 1991; Zhang, Rao, et al. 2007; Zhang et al. 2008). Urine-metabolized *R*,*R*-3,4-dehydro-*exo*-brevicomin and *S*-2-*sec*-butyl-4,5-dihydrothiazole work synergistically to attract females (Jemiolo et al. 1985). Female mice use quantitative variations in some of these male pheromone compounds to assess the quality of male mates. For example, increasing *E*-β-farnesene and *E*,*E*-α-farnesene levels in dominant male urine enhances its attractiveness to females (Jones and Nowell 1973; Harvey et al. 1989; Novotny et al. 1990; Liu et al. 2008).

Here, we aim to explore the relationship between pheromone composition, genetic correlates and mate choice in mice. From the framework outlined above, we predict that females use differences in urine odor to choose between healthy and immunodeficient nude males in both inbred and outbred strains; and that healthy and immunodeficient

nude males produce quantitatively and/or qualitatively varying pheromone compounds in voided urine. We tested these predictions using combined behavioral analyses of female mate choice and chemical analysis of both urinary and preputial gland volatile odorants of healthy and immunodeficient nude males. We used healthy or normal strains as odor detectors. Because *Foxn1* gene knockout female mice are athymic, T-cell deficient, and experience low pregnancy, they were assumed to be abnormal behaviorally and physiologically and so used as odorant donors only rather than odor detectors (Schorpp et al. 1997; Schlake 2001).

Materials and methods

Subjects

We used 6 CD-1 (ICR/albino), 6 CD-1 *nu*, 6 BALB/c, and 6 BALB/c *nu* male mice as odor donors and 12 CD-1 female mice and 12 BALB/c female mice as corresponding odor detectors. Twelve additional CD-1 females were used as odor recipients in habituation–dishabituation tests. The mice were at the age of 8 weeks and sexually inexperienced when obtained from a pathogen-free (SPF) facility (Weitong-Lihua Experimental Animal Company, Beijing, China). Males were housed individually and females in groups of 4 in standard 31.8-cm × 20.2-cm × 31.5-cm plastic cages. The housing room had a reversed 14:10 h light:dark photoperiod (light on at 19:00) and was maintained at 25 ± 1 °C. Standard mouse chow and tap water were provided ad libitum. Only reproductively active individuals (estrous females and scrotal males) were used. We determined female estrous cycles by vaginal smears. Mice serving as odor donors were housed under SPF conditions and the detector mice were under conventional conditions at our facilities at the Institute of Zoology, Chinese Academy of Science. This meant that for donor mice, the housing room 3.5 m × 5 m contained only sterilized feed, bedding, supplies, and equipment. All mice used as odor donors were kept in microisolator cages. All animal technicians and experimenters were required to wear sterilized clothing, shoes, gloves, masks, and caps at all times.

Scent collection and sample preparation

To collect urine, we placed each donor male in a clean mouse cage floored with a wire grid. When the animal urinated, the urine was immediately absorbed and transferred to a clean vial by a disposable glass capillary (i.d.: 1.8 mm; length: 15 cm). All urine was collected during the dark phase. To obtain the preputial glands, we euthanized the mice by cervical dislocation, and immediately dissected and weighed the paired preputial glands. The yellowish preputial gland secretion (PGS) was then collected by squeezing the glands and collecting PGS in a clear vial. PGS was kept at –20 °C until extraction. Solvent extraction involved mixing 250 μL dichloromethane (purity > 99.5%) with 250 μL urine. We weighed the PGS and added dichloromethane into the vial in the proportion of 1 mg PGS in 10 μL dichloromethane. We let this sit for 12 h at 0 °C and then used the bottom phase (i.e., the layer with dichloromethane) for chemical analysis (Zhang, Rao, et al. 2007).

Behavioral 2-choice test

Two-choice tests were conducted following a simplified binary choice test that has repeatedly been shown to be effective for pheromone behavioral assays (e.g., Zhang, Sun, et al. 2007; Zhang et al. 2008). In this method, the preference of female mice to 2 aqueous scent samples (CD-1 vs. CD-1 *nu* male urine and BALB/c vs. BALB/c *nu* male urine) was tested during the dark phase to coincide with nocturnal activity in this species. Immediately before each trial, a test mouse in its home cage was transferred to the test room under dim light. Scent samples were presented to the mouse by a disposable glass capillary (i.d 1.8 mm, o.d. 2.0, length 15 cm) containing a 2-μL sample aliquot. The opening of the capillary was sealed by odorless gum so as to suspend the aqueous sample inside the capillary 1 cm away from the sample-containing end. The sample-containing end of the capillary was presented to a female mouse, and the other end was held by a tester wearing disposable plastic gloves. The 2 capillaries were lowered through the wire lid and kept approximately 2 cm apart. Two aqueous samples were presented simultaneously to the test subject for 3 min after showing an initial sniffing response. The time that the mouse spent sniffing within 1 cm from the tip and licking the end of the capillary was recorded using stopwatches for both treatments. A mouse was used only once a day. Subjects whose investigation time was less than 1 s on a day were excluded for that day. With this method, individual urine samples of the 6 CD-1 males were randomly paired with those of the six CD-1 *nu* males and then presented to female recipients. Each urine sample was used twice. This bioassay has been proven to be sensitive enough to test the differences in the attractiveness of urine from dominant and subordinate males to females (Liu et al. 2008).

Habituation–dishabituation test

We exposed each CD-1 female mouse recipient to the urine from 1 CD-1 male on a series of 4 trials, each lasting 3 min with a 2-min interval between consecutive trials, and measured the time females spent investigated this odor. The other odor from 1 CD-1 *nu* male was presented on the fifth trial. For each trial, a fresh odor sample was placed in a new glass capillary following the protocols above. Any subject that did not respond to the odor during the first 3 mins was excluded for the day (Pankevich et al. 2004; Zhang et al. 2008).

Chemical assay

Gas chromatography and mass spectrometry (GC–MS) analysis was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective

Detector (NIST 2002 Library). The GC was equipped with an HP5MS glass capillary column (30 m long, i.d.: 0.25-mm × 0.25-μm film). The carrier gas was helium (1.0 mL/min.). The injector temperature was set at 230 °C. The oven temperature was set initially at 100 °C (for PGS), heated by 5 °C/min to 225 °C (for urine, the initial temperature was set at 50°C, which was heated by 5 °C/min to 105 °C), and then ramped by 5 °C/min to 230 °C, at which the oven temperature was maintained for 15 min to clean the column. Electron impact ionization was at 70 eV. Transfer line temperature was set at 280 °C. Scanning mass ranged from 30 to 350 amu. For each run, 1 μL of sample was injected in a split mode (10:1) for PGS or a splitless mode for urine.

The components were identified by comparing their GC retention indices and mass spectra to the reference compounds and literature data (Novotny et al. 1999; Zhang, Rao, et al. 2007). The standard compounds were obtained from ACROS Inc. (Geel, Belgium).

To quantify relative abundance (e.g., percent GC area) for each compound in a sample, we used the GC peak area of a particular compound as the absolute abundance and converted the peak area into the percentage of the summed peak areas as the relative abundance (Sun and Müller-Schwarze 1998a, 1998b; Zhang, Rao, et al. 2007).

Statistical analyses

Data distribution was first tested for normality using a Kolmogorov–Smironov test. For paired samples, we used Wilcoxon signed-rank test for nonnormal data and paired *t*-test for normal data. For independent samples, we used Mann–Whitney test for nonnormal data and independent *t*-test for normal data. Significance was accepted when $P < 0.05$. All statistical tests were carried out using SPSS version 13.0.

Ethical note

The procedures of animal care and use in this study fully complied with the legal requirements of China and were approved by the Animal Use Committee of the Institute of Zoology, Chinese Academy of Science.

Results

Sex attractiveness of voided urine from normal and nude male mice

Binary choice tests revealed that BALB/c females showed a significant preference for BALB/c urine over BALB/c *nu* urine ($Z = 2.118$, $P = 0.034$) (Figure 1A). Habituation/dishabituation tests revealed that the time spent investigating by CD-1 females during the fourth presentation of CD-1 male urine was lower when compared with the first ($N = 12$, $Z = 3.059$, $P = 0.002$), whereas the investigation time during the fifth presentation of CD-1 *nu* male urine was longer compared with the fourth ($N = 12$, $Z = 2.490$, $P = 0.013$) (Figure 1B). However, sexually naive CD-1 females did not show an overt preference for voided urine samples from either CD-1 or CD-1 *nu* males ($t = 0.262$, $P = 0.798$).

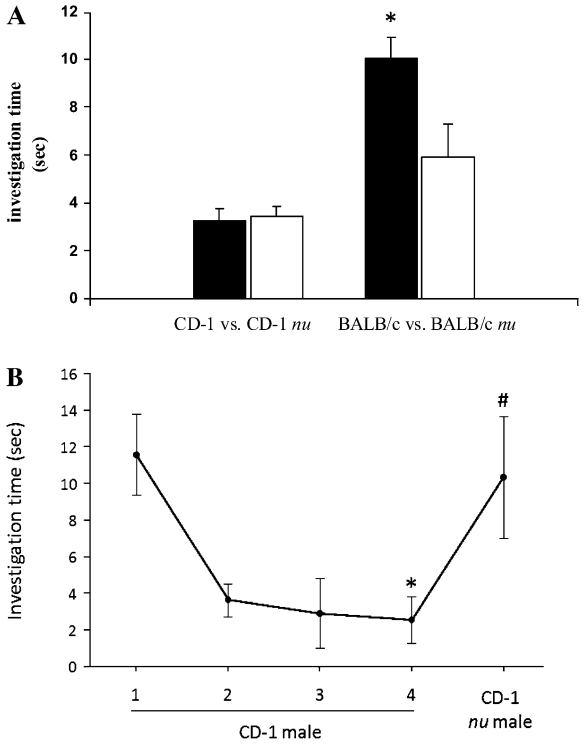

Figure 1 **(A)** Time in seconds (mean ± standard error, $N = 12$) spent by same-strain females investigating urine odor of CD-1 versus CD-1 *nu* or BALB/c versus BALB/c nu male mice (paired *t*-test, $*P < 0.05$). **(B)** Habituation–dishabituation test of the ability of CD-1 female mice to discriminate between CD-1 male mouse urine and CD-1 *nu* male mouse urine. Paired-samples *t*-test or Wilcoxon signed-ranks test was used. $*P < 0.05$ between CD-1 male urine 1 and 4, $\#P < 0.05$, between CD-1 male urine 4 and CD-1 nu male urine using Wilcoxon matched-pairs signed-rank test, $N = 12$).

The influence of *Foxn1* knockout on male organs

Body weight ($t = 11.11$, $P < 0.001$) and preputial gland weight ($t = 4.382$, $P = 0.001$) were both higher in CD-1 than in CD-1 *nu* male mice. However, the relative weight of the spleen ($t = 2.856$, $P = 0.017$) was higher in CD-1 *nu* males than in CD-1 males. The relative weight of the preputial gland did not differ between males of these 2 strains (Table 1). On the contrary, body weight and preputial gland weight did not differ between BALB/c and BALB/c *nu* male mice, but the relative weight of the preputial glands was larger in BALB/c males than in BALB/c *nu* males (Table 1). The relative ($Z = 2.882$, $P = 0.004$) and absolute weight ($Z = 2.882$, $P = 0.004$) of the spleen was significantly heavier in BALB/c *nu* males compared with BALB/c males (Table 1).

The influence of *Foxn1* knockout on male odor composition

A total of 23 compounds were detected in PGS. Three compounds had a higher relative abundance (compounds 9, 10,

Table 1 Body weight and mass of selected organs of healthy and *Foxn1*-knockout (*nu*) male mice (mean ± standard deviation [SD], $N = 6$)

Physiological parameters		Outbred mice		Inbred mice	
		Healthy CD-1	CD-1 *nu*	Healthy BALB/c	BALB/c *nu*
Body weight (g)		37.33 ± 2.503[a]	24.17 ± 1.472[a]	22.45 ± 2.427	23.37 ± 0.258
Preputial glands	Weight (mg)	133.4 ± 27.05[a]	84.58 ± 3.325[a]	66.55 ± 12.63	56.53 ± 5.627
	RW	3.550 ± 0.497	3.509 ± 0.217	2.954 ± 0.395[b]	2.420 ± 0.243[b]
Testes	Weight (mg)	206.7 ± 84.57	172.5 ± 25.48	149.1 ± 11.49	163.9 ± 18.11
	RW	5.556 ± 2.320	7.144 ± 0.978	6.667 ± 0.388	7.023 ± 0.841
Adrenals	Weight (mg)	5.717 ± 2.122	3.500 ± 1.383	2.817 ± 0.471	2.950 ± 0.505
	RW	0.152 ± 0.055	0.143 ± 0.050	0.126 ± 0.024	0.126 ± 0.022
Spleen	Weight (mg)	99.83 ± 16.72	90.68 ± 19.94	74.90 ± 10.33[b]	137.0 ± 77.23[b]
	RW	2.667 ± 0.351[a]	3.769 ± 0.877[a]	3.340 ± 0.365[b]	5.838 ± 3.205[b]

RW = relative weight (mg/g body weight). Mean values in a row marked by the same superscript letters are significant at the 0.05 level using an independent *t*-test or Mann–Whitney *U* test.

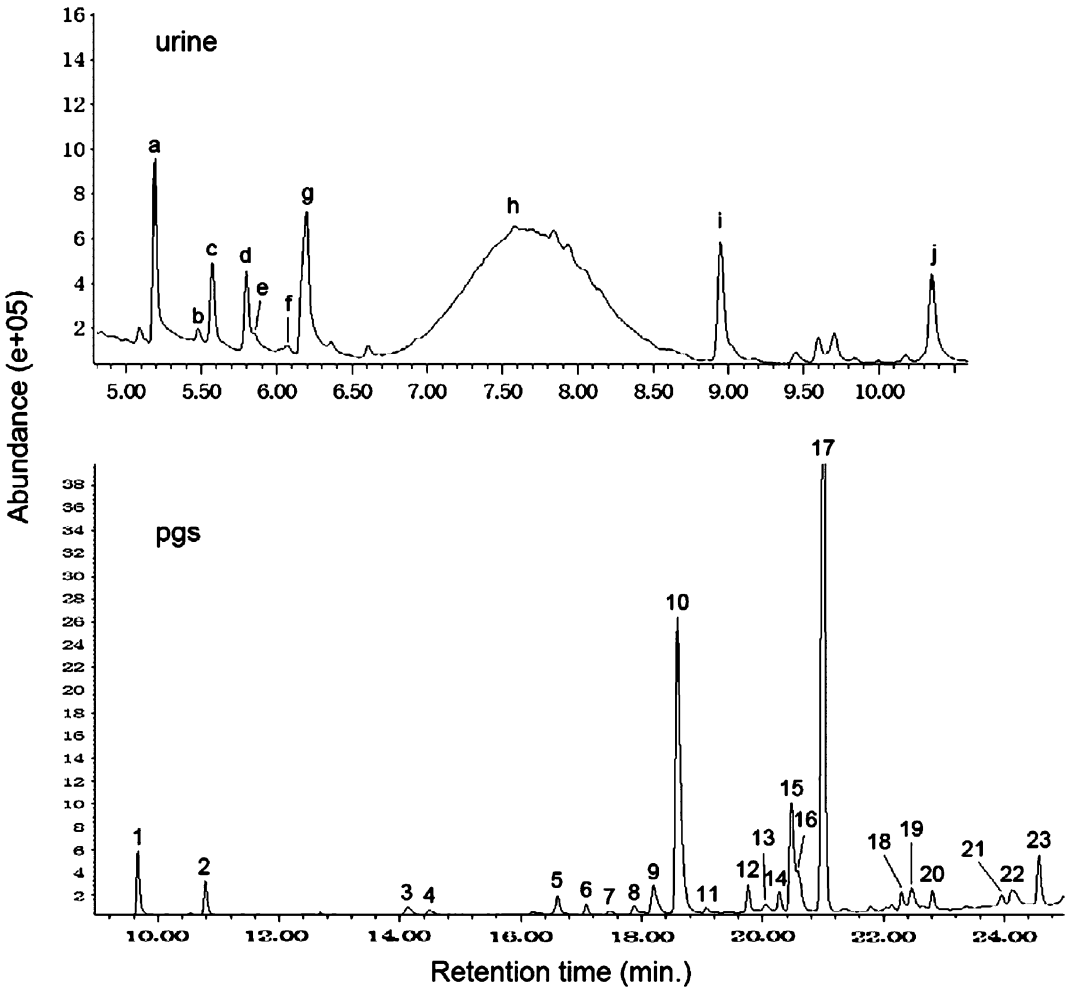

Figure 2 Representative gas chromatograms of urine (top panel) and PGSs (bottom panel) from CD-1 male mice. The letters and numbers used to label GC peaks correspond to the peak letters and numbers in Table 2 for urine and Table 3 for PGS.

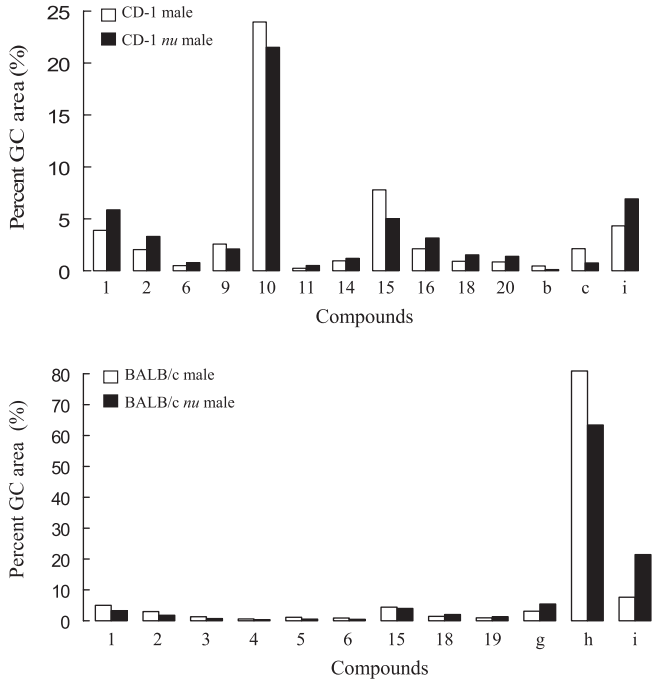

Figure 3 Summary of the glandular (numbered) and urinary (lettered) volatile compounds showing significant differences in percent GC areas between healthy (white bar) and immunodeficient (black bar) CD-1 (top panel) or BALB/c (bottom panel) male mice. Compound numbers and letters correspond to those used in Tables 2 and 3.

and 15) in CD-1 males than in CD-1 *nu* males, whereas 8 (compounds 1, 2, 6, 11, 14, 16, 18, and 20) were significantly higher in CD-1 *nu* males than in CD-1 males (Figures 2 and 3, Table 2). Seven compounds (compounds 1–6 and 15) were significantly higher, and 2 of them (compounds 18 and 19) significantly lower in BALB/c males compared to BALB/c *nu* males.

We detected 10 urine-metabolized volatiles in CD-1 and CD-1 *nu* male urine (Figures 2 and 3, Table 3). Among them, compounds b and c were significantly higher, whereas compound h was lower ($t = 2.159$, $P = 0.056$) in relative abundance in CD-1 males than in CD-1 *nu* males (Figure 3, Table 3). On the other hand, across the 9 urine volatiles detected in BALB/c and BALB/c *nu* males, compounds g and h were significantly higher, but compound i was significantly lower in BALB/c males than in BALB/c *nu* males (Figure 3, Table 3).

Discussion

Our behavioral experiments revealed that inbred BALB/c females but not outbred CD-1 females preferred the urine of control/healthy males over that of immunocompromised males despite measured differences in the qualities of their urine. Although knocking out the *Foxn1* gene affected urinary pheromone composition and relative abundance in the *nu* strains for both CD-1 and BALB/c male mice, no compound from the preputial gland or urine was eliminated entirely. Although this quantitative change of urine-borne volatiles, theoretically, could provide a clear olfactory cue for females to detect and respond to differences in males that only differ in a single gene, only inbred mice demonstrated this pattern. It is possible that for outbred CD-1 and CD-1 *nu* mice compensatory changes such as increases in some urinary and PGS compounds that are involved in female attraction and decreases in others may offset one another. A compensatory mechanism would explain why outbred females showed no preference for urinary from control or immunecompromised males. Our results provide an interesting case where male pheromones preferred by females do not reflect males' health and could not function as an honest signal. Interestingly, CD-1 females were able to discriminate between the urine of CD-1 and CD-I *nu* males in our habituation/dishabituation test, suggesting that urine-borne volatiles show interstrain differences that could be detected by CD-1 females as 2 kinds of odortypeodors but did not code sufficient information about male quality to alter female response (Pankevich et al. 2004; Liu et al. 2009).

Among heightened compounds in the preputial glands of immunodeficient CD-1 *nu* male mice, *E*-β-farnesene and *E*,*E*-α-farnesene are known male pheromones attractive to females (Jemiolo et al. 1991). 1-Tetradecanol acetate, 1-pentadecanol acetate, a branched 1-hexadecanol acetate, and *Z*-11-hexadecenol acetate are putative male pheromones with similar effects on females. Among heightened compounds in the preputial glands of healthy CD-1 male mice, hexadecanol is a male pheromone compound attractive to females synergistically with hexadecyl acetate. *Z*-9-hexadecenol and an isomer of *Z*-11-hexadecenol acetate are putative male pheromones, which are attractive to females (Zhang, Rao, et al. 2007; Zhang et al. 2008). Of the urinary metabolites, *R*,*R*-3, 4-dehydro-*exo*-brevicomin, which was higher in CD-1 *nu* than in CD-1 mice, is a confirmed male pheromone component attractive to females when presented synergistically with *S*-2-*sec*-butyl-4,5-dihydrothiazole (also a urine metabolite) (Jemiolo et al. 1985). Urinary 2-heptanone and *E*-5-hepten-2-one, both higher in CD-1 than in CD-1 *nu* mice, have been shown to have higher ratios in female mice than male mice, indicative of possible male attractants at their physiological levels in females and of possible female attractants at their physiological levels in males (Men ZJ, Zhang YH, Huo Y, Zhang JX, unpublished data). This kind of opposing effect of pheromone components in a dosage-dependent manner has been exemplified with preputial gland–derived hexadecanol and hexadecyl acetate in mice (Zhang et al. 2008).

Confirmed and possible female attractants were enhanced in both healthy and immunocompromised BALB/c males. The preputial gland–secreted *E*-β-farnesene and *E*,*E*-α-farnesene, both confirmed female attractants, and 5 other possible female attractants from males including *Z*-7-tetradecenol, 1-tetradecanol, *Z*-5-tetradecenol acetate, 1-tetradecanol acetate, and an isomer of *Z*-11-hexadecenol

Table 2 Relative abundance of preputial gland volatiles in healthy and *Foxn1*-knockout (*nu*) male mice (mean ± SD, $N = 6$)

GC peak no.	Compounds[a]	Outbred mice		Inbred mice	
		Healthy CD-1	CD-1 *nu*	Healthy BALB/c	BALB/c *nu*
1	*E*-β-Farnesene*	3.890 ± 0.756[a]	5.865 ± 0.570[a]	4.998 ± 0.872[b]	3.292 ± 1.417[b]
2	*E*,*E*-α-Farnesene*	2.035 ± 0.443[a]	3.303 ± 0.419[a]	2.952 ± 0.528[b]	1.812 ± 0.950[b]
3	*Z*-7-Tetradecenol	0.767 ± 0.232	0.871 ± 0.348	1.297 ± 0.404[b]	0.685 ± 0.327[b]
4	1-Tetradecanol*	0.439 ± 0.141	0.545 ± 0.254	0.612 ± 0.209[b]	0.359 ± 0.173[b]
5	*Z*-5-Tetradecenol acetate*	0.960 ± 0.291	1.196 ± 0.332	1.108 ± 0.184[b]	0.520 ± 0.349[b]
6	1-Tetradecanol acetate*	0.495 ± 0.178[a]	0.774 ± 0.199[a]	0.889 ± 0.107[b]	0.462 ± 0.240[b]
7	*Z*-11-Hexadecenol	0.211 ± 0.052	0.275 ± 0.086	0.176 ± 0.044	0.175 ± 0.101
8	1-Hexadecanol (branched)	0.411 ± 0.142	0.571 ± 0.116	0.414 ± 0.032	0.360 ± 0.135
9	*Z*-9-Hexadecenol	2.461 ± 0.262[a]	2.100 ± 0.237[a]	1.570 ± 0.219	1.321 ± 0.497
10	1-Hexadecanol*	23.95 ± 1.732[a]	21.52 ± 1.710[a]	18.69 ± 1.550	16.29 ± 5.226
11	1-Pentadecanol acetate	0.236 ± 0.144[a]	0.508 ± 0.076[a]	0.406 ± 0.045	0.347 ± 0.057
12	*Z*-9-Hexadecenol acetate	1.117 ± 0.386	1.129 ± 0.164	1.001 ± 0.075	0.914 ± 0.142
13	1-Heptadecanol (branched?)	0.519 ± 0.123	0.728 ± 0.259	0.534 ± 0.048	0.602 ± 0.098
14	1-Hexadecanol acetate (branched)	0.954 ± 0.166[a]	1.202 ± 0.100[a]	1.258 ± 0.072	1.146 ± 0.137
15	Isomer of *Z*-11-Hexadecenol acetate	7.790 ± 0.613[a]	5.017 ± 0.717[a]	4.431 ± 0.262[b]	3.978 ± 0.359[b]
16	*Z*-11-Hexadecenol acetate	2.118 ± 0.799[a]	3.148 ± 0.751[a]	2.325 ± 0.433	2.188 ± 0.373
17	1-Hexadecanol acetate*	42.46 ± 0.578	41.65 ± 2.763	49.49 ± 3.645	54.30 ± 7.749
18	1-Heptadecanol acetate (branched)	0.923 ± 0.133[a]	1.530 ± 0.410[a]	1.434 ± 0.117[b]	2.024 ± 0.536[b]
19	1-Octadecanol*	1.673 ± 0.356	1.410 ± 0.264	0.976 ± 0.124[b]	1.339 ± 0.297[b]
20	1-Heptadecanol acetate	0.853 ± 0.251[a]	1.403 ± 0.396[a]	0.896 ± 0.098	1.249 ± 0.320
21	Dodecyl octanoate	0.863 ± 0.288	0.836 ± 0.070	0.978 ± 0.072	1.001 ± 0.159
22	*Z*-7-Octadecenol acetate	1.696 ± 0.287	1.577 ± 0.276	0.868 ± 0.091	1.470 ± 0.627
23	Octadecanol acetate	3.180 ± 0.541	2.846 ± 0.460	2.697 ± 0.227	3.467 ± 1.120

Compounds marked * were definitively identified through comparison with authentic analogs, remaining compounds were identified using mass library (NIST 2002) and published studies (Novotny et al. 1999; Zhang, Rao, et al. 2007). Mean values in a row marked by the same superscript letters are significant at the 0.05 level using an independent *t*-test or Mann–Whitney *U* test.

acetate were enhanced. Meanwhile, the urinary dimethyl sulfone and 6-hydroxy-6-methyl-3-heptanone increased, but *R*,*R*-3,4-dehydro-*exo*-brevicomin decreased, in BALB/c males. The relatively larger preputial glands in BALB/c males might emit more pheromones into voided urine and also contribute to urine attractiveness for females. Unlike the crossin-creases in male pheromone compounds (or their candidates) in the urine of CD-1 and CD-1 *nu* males, most urine-borne odor compounds increased in healthy BALB/c mice as compared with BALB/c *nu* mice. Thus, according to our behavioral results, BALB/c females might prefer particular olfactory cues in choosing healthy BALB/c males over the immunocompromised BALB/c *nu* males. The involvement of at least 2, and possibly more, pheromone compounds used in decision making for mate choice in females also suggests that no single compound alone could serve as an honest signal, which had been expected to be associated with the level of immunocompetence.

Chemical signals, like visual and acoustic signals, can evolve in an exaggerated way to become honest indicators of a male disease resistance (Penn and Potts 1998). Because mouse behavior and physiology are jointly affected by single gene mutations and other background genes (Logue et al. 1997; Kelly et al. 1998; Võikar et al. 2001), differences in the effects of *Foxn1* knockout on urinary volatile composition in CD-1 *nu* and BALB/c *nu* males might be attributable to differences in the level of genetic variation between these inbred and outbred strains. For example, susceptibility to endocrine disruption is related to genetic variability in mice (Spearow et al. 1999), and therefore, *Foxn1* knockout–induced hormonal alterations might have different results

Table 3 Relative abundance of urinary volatiles in healthy and *Foxn1*-knockout t (*nu*) male mice (mean ± SD, $N = 6$)

GC peak no.	Compounds[a]	Outbred mice		Inbred mice	
		Healthy CD-1	CD-1 *nu*	Healthy BALB/c	BALB/c *nu*
a	*Z*-5,5-Dimethyl-2-ethylidenetetrahydrofuran	6.493 ± 8.000	11.05 ± 19.55	2.477 ± 0.653	0.438 ± 0.179
b	2-Heptanone*	0.468 ± 0.281[a]	0.127 ± 0.121[a]	0.018 ± 0.010	0.020 ± 0.011
c	*E*-5-hepten-2-one	2.139 ± 0.740[a]	0.749 ± 0.291[a]	0.066 ± 0.030	0.071 ± 0.014
d	*E*-5,5-Dimethyl-2-ethylidenetetrahydrofuran	1.230 ± 0.663	1.316 ± 0.700	0.941 ± 0.200	0.806 ± 0.175
e	*Z*-2-pentenyl acetate	0.354 ± 0.325	0.293 ± 0.620	—	—
f	Unknown	1.156 ± 1.273	1.232 ± 1.130	0.258 ± 0.129	0.336 ± 0.120
g	Dimethyl sulfone*	3.548 ± 1.436	5.109 ± 2.198	3.111 ± 0.743 [b]	5.409 ± 1.533 [b]
h	6-Hydroxy-6-methyl-3-heptanone and 5,5-dimethyl-2-ethyl-tetrahydrofuran-2-ol	71.22 ± 12.73	61.76 ± 20.919	80.87 ± 1.905 [b]	63.39 ± 12.37 [b]
i	*R*,*R*-3,4-dehydro-*exo*-brevicomin	4.320 ± 1.000[a]	6.923 ± 3.219[a]	7.599 ± 1.122[b]	21.43 ± 9.542[b]
j	*S*-2-*sec*-butyl-4,5-dihydrothiazole	9.344 ± 6.648	5.633 ± 1.232	4.659 ± 1.195	6.338 ± 2.095

Compound marked * was definitively identified through comparison with authentic analogs, other compounds were identified using mass library (NIST 2002) and literature (Novotny et al. 1999; Zhang, Rao, et al. 2007). Mean values in a row marked by the same superscript letters are significant at the 0.05 level (c: $P = 0.056$), using an independent *t*-test or Mann–Whitney *U* test. Compounds were identified by using mass library (NIST 2002) and literature (Zhang, Rao, et al. 2007). * $P < 0.05$; ** $P < 0.01$.

in mediating pheromone composition between CD-1 and BALB/c male mice.

It is well documented in mice that females show mate preferences for genetically dissimilar males, whose sexually selected signals indicate high genetic quality. Genetic similarity can act as a kin indicator and interact with mate choice processes and decisions by female mice (Isles et al. 2001; Roberts and Gosling 2003). The good-genes-as-heterozygosity hypothesis states that females, especially inbred females, may choose mates genetically different from themselves producing offspring of greater heterozygosity (Isles et al. 2001; Ilmonen et al. 2009). Inbred females may gain a greater benefit from this strategy than outbred females (Ilmonen et al. 2009). Earlier studies with inbred congenic mouse lines show that females not only choose males with non–self-MHC types (genetic nonsibling or not own strain) but also avoid mating with males carrying MHC genes of their foster family (social sibling). This suggests that MHC-dependent familial imprinting potentially provides an effective mechanism for avoiding mating with close kin (Penn and Potts 1998). It has been observed that BALB/c females chose the urine odor of C57B/c males over that of their own strain males and vice versa (Ilmonen et al. 2009; unpublished data). However, in the current study, inbred BALB/c females showed a preference for males of their own strain (e.g., normal BALB/c), suggesting that females avoided immunocompromised mates as induced by knocking out the *Foxn1* gene and that perhaps genetic dissimilarity was ranked lower than parasite load or immunocompetence in mate choice.

Fitness in many organisms declines with increasing levels of homozygosity, resulting in inbreeding depression, which poses an important selective force on reproductive behavior (Pusey and Wolf 1996; Keller and Waller 2002). Deleterious traits reflected by male odor and alterations of pheromone composition might be revealed by the synergetic effect of inbreeding depression and immunodeficiency. In our study, immunodeficiency induced by knocking out the *Foxn1* gene effectively suppressed the attractiveness of the urinary odor of inbred BALB/c mice. Our more recent results (unpublished data) from dexamethasone-induced immunodeficiency of inbred and outbred mice also confirmed such a synergistic effect. In combination, it appears that for mice, the ICHH holds true only under circumstances of little genetic variation and a high level of inbreeding seems to be a prerequisite for the ICHH for olfactory signals with multiple components. In outbred strains, different genetic-based pheromone components would likely undergo change indifferently, increasing in some while decreasing in others. This would necessarily result in the counteraction of these components in healthy and *Foxn1* knockout CD-1 males when attracting females as shown here. Our argument that the ICHH may not be universally applicable now needs to be closely scrutinized in other species and experiments.

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