

Scent-marking displays provide honest signals of health and infection

Sarah M. Zala,^a Wayne K. Potts,^b and Dustin J. Penn^a

^aKonrad Lorenz Institute for Comparative Ethology, Austrian Academy of Sciences, Savoyenstrasse 1a, 1160 Vienna, Austria, and ^bDepartment of Biology, 257 S. 1400 E., University of Utah, Salt Lake City, UT 84112, USA

Males of many species produce scent marks and other olfactory signals that function to intimidate rivals and attract females. It has been suggested that scent marks provide an honest, cheat-proof display of an individual's health and condition. Here we report several findings that address this hypothesis in wild-derived house mice (*Mus musculus domesticus*). (1) We exposed males to female odor, which induces an increase in testosterone, and found that sexual stimulation significantly increased the males' scent-marking and the attractiveness of their scent marks to females. (2) We challenged sexually stimulated males with a nonreplicating strain of bacteria (*Salmonella enterica* C5TS) to activate immunity and found that this significantly decreased the males' scent-marking and the attractiveness of their scent marks to females. (3) We collected scent marks from infected and sham-infected males when they were sexually stimulated or not, and we found that females could significantly discriminate the scent marks of infected versus control males, but only when the males were sexually stimulated. Taken together, our results indicate that male mice modulate their scent-marking display depending on their health and perceived mating opportunities. Increased scent marking enhances males' attractiveness to females, scent marks provide an honest indicator of bacterial infection (and perhaps immune activation), and females are able to assess the health of males more easily when males mark at a high rate. **Key words:** honest signaling theory, house mice, immunocompetence handicap hypothesis, *Mus*, parasite-mediated sexual selection, *Salmonella*, testosterone. [*Behav Ecol* 15:338–344 (2004)]

The elaborate secondary sexual traits used by males to attract females in many species provide honest indicators of a male's quality, including their parasite load and disease resistance (Clayton, 1991; Hamilton and Poulin, 1997), as predicted by Hamilton and Zuk (1982). The handicap theory of honest signaling argues that elaborate sexual characters provide honest, cheat-proof indicators of an individual's condition and quality because only high-quality individuals can afford to pay the costs of these traits (Zahavi, 1975). Similarly, the immunocompetence handicap (IH) hypothesis argues that secondary sexual traits honestly reflect resistance to infectious diseases because only the disease-resistant males can afford the high testosterone required to develop these traits due to the immunosuppressive effects of this steroidal hormone (Folstad and Karter, 1992; Wedekind and Folstad, 1994). To test these ideas, sexual selection researchers have generally focused on the colorful ornaments of birds and fish, whereas mammals and other visually drab animals are often presumed to lack conspicuous secondary sexual characters. Yet, as Darwin recognized, the scent glands, volatile olfactory signals, and scent-marking behaviors produced by male mammals are secondary sexual traits that probably evolved through sexual selection (Blaustein, 1981). The goal of our study was to investigate whether the scent marks of male house mice (*Mus musculus domesticus*) provide a sexual courtship display that reflect an individual's health and infection status to prospective mates.

Scent marking is a testosterone-mediated, sexually dimorphic behavior that plays an important role in mating and other social interactions in house mice and many other species (Brown, 1979; Eibl-Eibesfeldt, 1950; Ralls, 1971;

Thiessen and Rice, 1976). Male house mice scent mark by depositing many small urine spots on their territory when they become socially dominant (Desjardins et al., 1973), and scent marking plays a role in male–male interactions, including competitor assessment (Gosling, 1982; Gosling and Roberts, 2001; Hurst, 1990). Several lines of evidence indicate that scent marks also play a role in mate choice. For example, male mice also scent mark at a high rate when they encounter females (Bronson, 1979; Maruniak et al., 1974). Females are attracted to male scent marks (Hurst, 1990), and especially those of dominant (Drickamer, 1992; Jones and Nowell, 1974) and competitive males (Rich and Hurst, 1998). This is because males produce various androgen-mediated chemosensory signals, including major urinary proteins (MUPs), volatile odorants, and pheromones, that are attractive to females (Bronson, 1976; Hurst et al., 1998; Kimura and Hagiwara, 1985; Mucignat-Caretta et al., 1998; Novotny et al., 1984). Therefore, it has been suggested that scent-marking functions as a sexual courtship display, analogous to the exaggerated visual displays that other male vertebrates use, to advertise their quality and condition to potential mates (Penn and Potts, 1998).

It has also been suggested that scent marking and other chemosensory signals provide cheat-proof displays that honestly advertise males' health and other aspects of quality and condition to rivals and potential mates (Gosling and Roberts, 2001; Penn and Potts, 1998). This is supported by evidence that scent marking is physiologically costly (Gosling et al., 2000) and attracts predators (Viitala et al., 1995). Moreover, several studies have found that odor cues from males' urine and scent marks provide an indicator of their health and infection status, and that females are less attracted to the scent of infected versus uninfected males (Kavaliers and Colwell, 1995a,b; Klein et al., 1999; Penn et al., 1998; Willis and Poulin, 2000). Infection-induced alterations in odor may be a pathological byproduct of infection, a consequence of parasite manipulation of the host, or an adaptive response to

Address correspondence to S. M. Zala, Konrad Lorenz Institute, Austrian Academy of Sciences, Savoyenstrasse 1a, A-1160 Vienna, Austria. E-mail: s.zala@klivv.oeaw.ac.at.

Received 13 December 2002; revised 23 May 2003; accepted 11 June 2003.

cope with infection. Males often lower their testosterone during infection (Hillgarth and Wingfield, 1997), which may function to reduce the immunosuppressive effects of this steroidal hormone (Folstad and Karter, 1992) or to divert energy and resources into immunity (Wedekind and Folstad, 1994). If scent marks honestly reveal health because disease-susceptible males cannot afford to maintain high testosterone, as predicted by the IH hypothesis, then activating immunity with foreign antigens or non-replicating pathogen (vaccines) should trigger a reduction in testosterone concentration, scent marking, and the attractiveness of scent marks. This prediction is supported by a recent study that found that injecting male mice with foreign antigens (sheep red blood cells) reduced the attractiveness of their bedding to females (Moshkin et al., 2002). However, it is unclear whether activating immunity alters males' scent-marking display.

Here we report several findings that support the hypothesis that scent marking provides a sexual courtship display that honestly indicates health and disease. First, to test whether increased scent marking enhances males' attractiveness to females, we exposed males to female urine to induce an increase in testosterone concentration (Batty, 1978; Bronson and Desjardins, 1982; James and Nyby, 2002; Kavaliers et al., 2001; Macrides et al., 1975, unpublished data). We found that such sexual stimulation increased the males' scent marking and the attractiveness of their scent marks to females. Second, to test whether scent marking is altered by immunological activation, we injected males with a non-replicating strain of bacteria (*Salmonella enterica* serovar Typhimurium C5TS) and found that this reduced males' scent marking and the attractiveness of their scent marks to females. Third, to test whether females can assess the health of males more effectively when males mark more, we compared the attractiveness of scent marks from males that were experimentally infected versus controls using males that were sexually stimulated or not. We found that females could only discriminate healthy versus infected males when the males were sexually stimulated and when the males marked at a high rate.

METHODS

Subjects and housing

We obtained scent marks from sexually mature wild male house mice (*M. musculus domesticus*), maintained in an outbred colony (Meagher et al., 2000). For subjects (smellers) in odor preference assays, we used adult virgin females of an outbred laboratory strain of mice (Swiss Webster). We chose a laboratory strain because in preliminary trials with the apparatus females of the laboratory strain seemed less stressed than wild female mice and because in this manner we could control for individual variation in females' responsiveness. Before the experiments, male and female mice were singly housed in acrylic cages (30 × 19 × 13 cm) containing pine bedding and paper towels for environmental enrichment. They were provided food (Harlan Teklad Rodent Chew), water ad libitum, and kept at a constant temperature (22 ± 2°C) under a 12:12 h light:dark cycle. The treatment and control mice in each experiment were closely age matched (usually within 30 days). All the experiments were conducted in the Department of Biology, University of Utah, and were approved by the local Institutional Animal Care and Use Committee.

Collecting scent marks for the odor preference assays

To collect males' scent marks, we placed one piece of sterile filter paper (7.5 × 7.5 cm) in their cages overnight (17–19

h) and then stored marked filter papers individually in zippered plastic bags at –70°C. Scent-marked papers contain cues from urinary scent marks and perhaps also from salivary marks (Lee and Ingersoll, 1979). Whenever we collected scent marks, we sexually stimulated males by placing one filter paper (2 × 2 cm) containing female urine in the male's cage overnight (17–19 h). We used 10 µl of female urine for experiments 1 and 3 and 3–5 µl for experiment 2 because we were running out of urine. We collected female urine by bladder palpation and pooled the samples over 7–20 days per female to control for variation due to estrus. We stored the urine at –70°C until needed. We never used the urine of a female more than once in any experiment. When we repeatedly collected scent marks of the same male with different treatments, we carefully controlled potential sequence effects during scent-mark collection.

Collecting and quantifying scent marks

To quantify males' scent-marking patterns, we placed large filter papers (26.5 × 16.5 cm) that covered the cage floor of singly housed males. We collected scent marks as described above, except we removed water, food, and bedding to eliminate other factors that might affect the papers. We quantified the males' scent-marking patterns by scanning the marked filter papers with a fluorescence scanner, a phosphor imager (the scent marks of mice fluoresce in ultraviolet light; Desjardins et al., 1973), and then used a digital imaging program (AlphaEase 5.0) to quantify the number of marks and the total area marked. We considered all marks on the paper as urinary scent marks, regardless of size, shape, or shade (e.g., Figure 4).

Odor preference assays

We used a simultaneous choice assay to test the odor preferences of virgin female mice (Swiss Webster). We did not check for estrus (thus being conservative) to avoid stressing the females before odor preference assays. Our odor preference experimental apparatus was composed of two boxes (acrylic cages), one "start" and one "test" chamber, with an acrylic cover to contain volatile odors, and connected by a plastic tube (5 cm long × 3 cm diam). Both chambers contained two smaller plastic hiding boxes (15 × 8 × 10 cm; 3 cm diam opening). Before each trial, we placed two matched scent-marked filter papers, one inside of each of the two hiding boxes of the test chamber (alternating the sides). Each paper was only used once. For choosing a pair of marked papers to test, we used the following conservative procedure. (1) We chose the two filter papers to be compared blindly, without seeing the scent-marked filter papers. (2) We discarded the filter papers if one or both was not visually marked (by holding the paper against a light) with at least one scent mark (comparing a nonmarked paper with a marked paper was not informative for the purpose of the experiment), and then chose another pair blindly. This procedure is very conservative, given that infected males and nonstimulated males mark less (see Results). (3) If we had different matching possibilities after choosing several pairs of papers, we also tried to match the papers for the amount of damage due to chewing from the males, the amount of water stains (from water bottles), and the amount of scent marking (comparing a totally chewed paper with an intact paper is not a useful comparison for the purpose of the experiment).

We introduced a female mouse to the start chamber for 10–15 min to habituate her to the new environment (same amount of time for every experiment). We began each trial by opening a remote-controlled door that allowed the

female to leave the start and enter the test chamber. Once the female entered the test chamber (usually within 1–3 min), we closed a second remote-controlled door (to prevent her from returning) and observed her behavior in the test chamber via a video camera and monitor (a blind surrounded the odor preference apparatus to avoid observer bias and intrusions). Naïve observers recorded which of the two hiding boxes the female entered first (initial preference), the number of times she entered each hiding box (number of visits), and the time she spent inside each box during the 7-min trial. Any bias that the females displayed we considered an odor preference. We never used a female more than once in an experiment, except in experiment 3, in which each female was tested twice (see below). After each trial the apparatus was washed with water and ethanol to remove residual odor traces.

Experimental infections

We used *Salmonella enterica* C5TS because it is a temperature-sensitive mutant strain that is non-replicating at 37°C *in vivo* (Hormaeche et al., 1981). This bacterium was developed as a vaccine, and it activates a variety of immune responses (Nauciel and Espinasse-Maes, 1992; Nauciel et al., 1985). We infected adult wild male mice with a 0.2-ml solution of 10⁵ bacteria, injected intravenously (intraorbitally), and sham-infected adult wild males with 0.2 ml phosphate buffer solution (PBS). We also used *Salmonella enterica* LT2, which is viable, but avirulent in wild mice. We infected adult wild males intraperitoneally with 10³ bacteria and sham-infected adult wild males with 0.2 ml PBS. These males were not infected only for these experiments; they were used for additional experiments (unpublished data).

Experiment 1. Odor preference assay and sexual stimulation

To test whether females are more attracted to the odor of males when they are sexually stimulated, we presented 16 females with scent-marked papers from 16 males. We collected the scent-marked papers as described above—10 papers from each male, 5 papers with and 5 without sexual stimulation (160 papers). The urine used for sexual stimulation was collected as described above, from 15 wild females. The females used in the odor preference assay could choose between two papers marked by the same male, one marked when the male was sexually stimulated and one marked when he was not (every trial used scent-marked papers from a different male).

Experiment 2. Odor preference assay and infection, with sexual stimulation

To test whether immune activation impairs the attractiveness of males' scent marks, we presented 28 females with scent-marked papers collected from bacterial-challenged (*Salmonella enterica* C5TS) and sham-infected males. We collected marked papers from 19 experimentally infected and 16 sham-infected male mice (9 papers per male), all during sexual stimulation (315 papers). The papers were collected 3–5 days after infection to allow time for specific as well as nonspecific immune responses to be activated. The urine used for sexual stimulation was collected as described above, from 15 wild females. When we presented scent-marked papers from the same males to a different female, we never offered the same pairwise combination of males.

Experiment 3. Odor preference assay and infection, with and without sexual stimulation

The males in experiment 2 were sexually stimulated during scent collection, which raises the possibility that the increased marking from sexual stimulation is necessary for females to detect such differences. Therefore, we tested the ability of females to distinguish infected versus control males with and without sexual stimulation. To test whether females more easily recognize infected males when males are sexually stimulated, we presented 37 females with scent marks from 37 pairs of males. For each pair of males, there was an infected (*Salmonella enterica* LT2) and a sham-infected mouse. Scent-mark papers were collected as described above (3–5 days after infection), during both sexual odor stimulation and during controlled (water) stimulation (two collections for each pair of males). The urine used for sexual stimulation was collected as described above, from 30 Swiss Webster. In the odor preference assay, the marks of each pair of males was compared twice (using the same female), once when the males' marks were collected during sexual stimulation and again when neither were stimulated (water control). To control for potential order effects, half of the females were presented with scent marks from stimulated males first, whereas the other half were offered the controls first.

Experiment 4. Scent-mark quantification and sexual stimulation

To compare scent-marking patterns of sexually versus non-sexually stimulated males and males exposed to male odor, we collected large filter papers as described above. We collected scent marks of 15 males, 3 times each (45 papers): once during sexual odor stimulation (with a mixture of urine from 10–15 wild females collected over time), once with water stimulation (control), and once with male urine stimulation (using a mixture of urine from 11 wild males collected over time). We randomized the order of the scent-mark collections to control for potential sequential effects (i.e., during the first collection we applied all three different stimuli to groups of five mice and then shifted the stimuli for the second and the third collection, so that every male was tested once with all three treatments).

Experiment 5. Scent-mark quantification and infection

To compare the scent-marking patterns of experimentally infected versus healthy males, we collected large filter papers as described above (3–5 days after infection). We obtained scent marks from 19 infected (*Salmonella enterica* C5TS) and 14 sham-infected males during sexual odor stimulation (as in experiment 4).

Statistical analyses

We used Systat (version 5.2.1) and JMP (version 3.2) to analyze the data. When the data were not normally distributed or the means had unequal variances, we performed nonparametric statistics. We used two-tailed tests (unless otherwise stated), directed tests when the direction of a test could be prescribed *a priori* (Rice and Gaines, 1994), and one-tailed tests when the outcome of the experiment was predicted from our previous data. The results are reported as means ± standard deviations, unless stated otherwise.

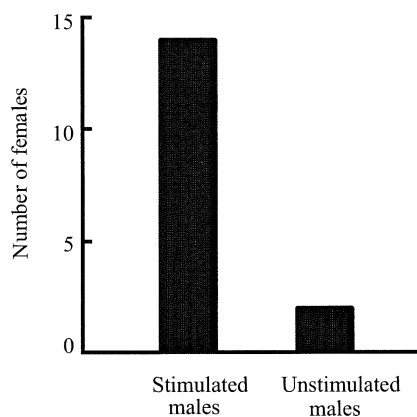


Figure 1
Number of females attracted to scent marks of males collected when they were sexually stimulated with female odor compared with the same male without sexual stimulation (initial preference; $n = 16$ females).

RESULTS

Experiment 1. Odor preference assay and sexual stimulation

When females were presented with scent marks from sexually stimulated males versus the scent marks from the same males collected when they were not stimulated, 14 of the 16 females first entered the hiding boxes containing the scent marks of the stimulated males (initial preference; binomial test, $n = 16$, $p = .004$; Figure 1). Number of visits (13.2 ± 4 sexually stimulated vs. 11.5 ± 3 control) and time (91.1 ± 36 s sexually stimulated vs. 76.5 ± 28 s control) spent in the hiding boxes were not significantly different, though there was a trend in the expected direction (Wilcoxon Signed-Ranks test, $n = 16$, $p = .34$ for number of visits and $p = .22$ for time in the box).

Experiment 2. Odor preference assay and infection, with sexual stimulation

Females were significantly less attracted to the marks of males experimentally challenged with the temperature-sensitive mutant bacteria, *Salmonella enterica* C5TS, than those of sham-infected males (number of visits; Wilcoxon Signed-Ranks test, $n = 28$, $p = .04$; Figure 2). Females' initial preference (16 of 28 preferred the uninfected marks) and time spent (measured in seconds) in hiding boxes (92.4 ± 31 sham and 90 ± 27 infected) did not differ significantly for the treatments (binomial test, $n = 28$, $p = .57$, and Wilcoxon Signed Ranks test, $n = 28$, $p = .64$, respectively).

Experiment 3. Odor preference assay and infection, with and without sexual stimulation

We repeated experiment 2, and we presented females with scent marks from infected versus sham-infected males (using *Salmonella enterica* LT2). Again, we found that females were significantly less attracted to the scent of infected males, but only if the scent marks were collected during sexual stimulation (initial preference; binomial test, $n = 37$, $p_{1\text{-tailed}} = .05$ with stimulation, $p = 1$ without stimulation; Figure 3). There was not a significant difference in the number of visits and time females spent in the boxes, for either scent collected during stimulation (Wilcoxon Signed-Ranks test, $n = 37$, $p_{1\text{-tailed}} = .26$ for visits; paired t test, $p_{1\text{-tailed}} = 0.2$ for time) or without

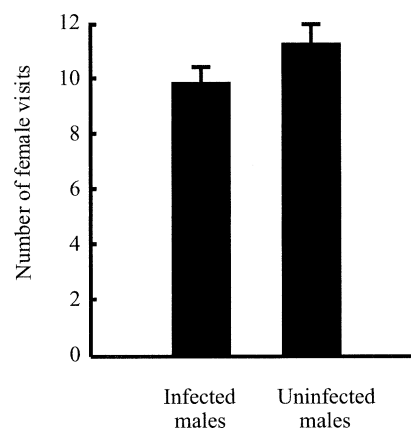


Figure 2
Number of females attracted to the scent marks of infected with the non-replicating, temperature-sensitive bacterial strain (*Salmonella enterica* C5TS) versus sham-infected males (number of visits; $n = 28$ females; mean \pm SE).

(Wilcoxon Signed-Ranks test, $n = 37$, $p = .54$ for visits; paired t test, $p = .75$ for time).

Experiment 4. Scent-mark quantification and sexual stimulation

When we examined the scanned images of the male's scent-marking patterns, we found that males increase the total number of scent marks when they are sexually stimulated (female urine) compared to when the same males are stimulated with water or male urine (Figure 4; Friedman test, $n = 15$, $df = 2$, $p = .03$). This effect was due to the males scent marking significantly more during sexual stimulation versus water control (Wilcoxon Signed-Ranks test, $n = 15$, $p = .01$). There were no statistically significant differences in the total number of marks when males were stimulated with female versus male urine (Wilcoxon Signed-Ranks test, $n = 15$, $p = .53$) or with male urine versus water (Wilcoxon Signed-Ranks test, $n = 15$, $p = .1$). Sexual stimulation did not change the total marked area (Friedman test, $n = 15$, $df = 2$, $p = .5$).

Experiment 5. Scent-mark quantification and infection

We found that during infection with *Salmonella* C5TS, males reduced the area marked (in pixels) on the paper (Mann-Whitney U test, $n = 33$, $p = .01$; Figure 5). The total number of marks (200.1 ± 237 infected and 270.6 ± 263 sham-infected) were not statistically significant different (Mann-Whitney U test, $n = 33$, $p = .32$).

DISCUSSION

The results of our experiments are generally consistent with the hypothesis that scent marks function as sexual courtship displays that provide honest indicators of health and disease (Penn and Potts, 1998). First, we found that male mice increased their scent marking when exposed to female odor (experiment 4) and that this manipulation increased the attractiveness of their scent marks to females (experiment 1). Sexual stimulation only had a significant influence on initial attraction of females, which may have been due to changes in both the quality (e.g., volatility), as well as the quantity of scent marks. Previous studies have found that males increase their scent marking when exposed to females, and it has been suggested that the presence of an estrous female is the most

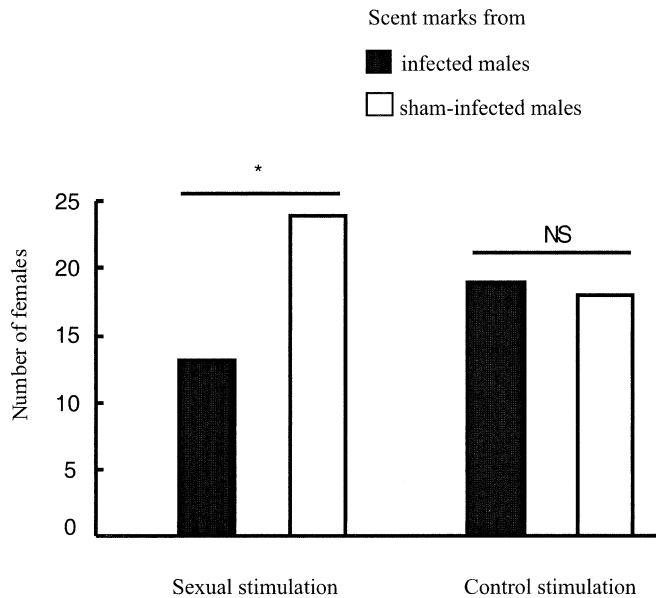


Figure 3

Number of females attracted to the scent marks of males collected when the males were experimentally infected with a viable but avirulent strain of *Salmonella* (LT2) versus sham-infected males. These comparisons were made using the same females with the same pair of males when the males were all sexually stimulated ($*p < .05$) and when the males were not sexually stimulated (NS). With sexual stimulation, 24 of 37 (65%) females first preferred the scent marks of the uninfected males. With the control stimulation (water), 18 of 37 (49%) first preferred the scent marks of the uninfected males.

important factor affecting males' scent-marking (Maruniak et al., 1974). Ours is the first study to our knowledge to show that female odor is sufficient to induce increased scent marking, and to show that increased scent marking enhances the attractiveness of males' scent marks to females.

Second, we found that activating immunity of males with a non-replicating strain of bacteria decreased their scent marking (experiment 5) and the attractiveness of their scent marks to females (experiment 2). The impact of infection on the attractiveness of scent marks was weak, but the effect was significant and repeatable, even though we used avirulent bacterial strains. Infection with the non-replicating bacterial strain reduced all measurements of sexual attractiveness of scent marks, though only the number of visits was significantly different. Our finding that scent marks reveal bacterial infection was supported in experiment 3 using a more virulent *Salmonella* strain, but the only significant effect was on initial attraction rather than on number of visits (this suggests that volatile odors were mainly influenced). We cannot explain this difference between experiments, though it may simply be a sample-size problem (insufficient power), or the different strains may have different effects on the chemical composition of scent marks. Previous studies have shown that the sexual attractiveness of a male's scent is reduced by a variety of infectious agents (Kavaliers and Colwell, 1995a,b; Klein et al., 1999; Penn et al., 1998; Willis and Poulin, 2000). Our study provides the first evidence that a bacterial infection reduces the attractiveness of males' scent marks, and it is the first to show that infection alters males' scent-marking patterns. Because we used a non-replicating bacterial strain, our study provides further evidence that immune activation is sufficient to reduce the sexual attractiveness of males' scent marks (Moshkin et al., 2002). These findings are also consistent with the immunocompetence handicap hypothesis, and they argue

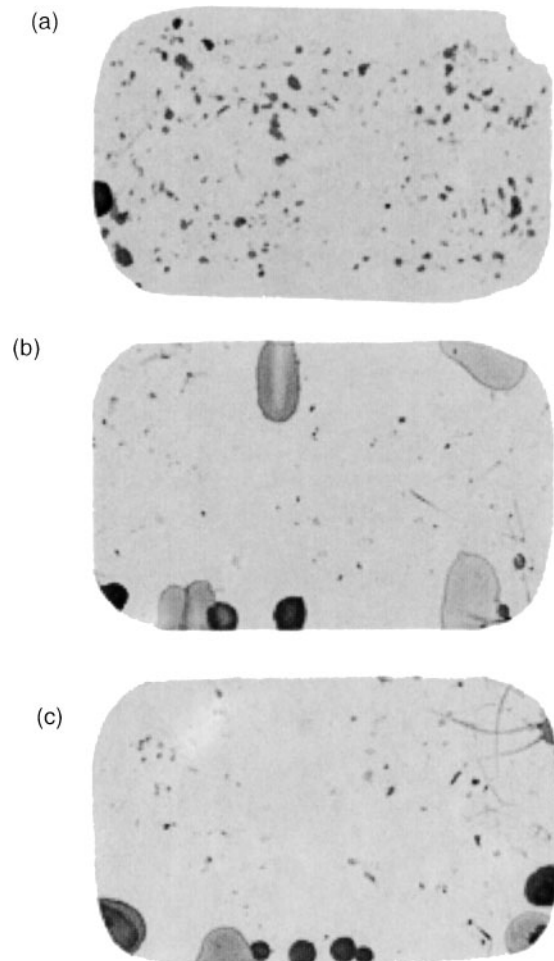


Figure 4

An example of the scanned images of three large filter papers (26.5 × 16.5 cm) collected for quantifying males' scent marks. These filter papers were collected from the same male mouse after being exposed to (a) female urine, (b) male urine, or (c) water control.

against the hypothesis that scent marks reveal infection simply as a pathological by-product of disease.

Third, we found that females were able to discriminate the scent marks of infected versus uninfected males more easily when the males were sexually stimulated and marked more (experiment 3). This indicates that the high rate of scent marking triggered by sexual stimulation enables females to assess males' health more effectively. Because a male's scent is not constitutively expressed and varies depending on their perceived mating opportunities, studies that collect scent from males that are not sexually stimulated may underestimate the amount of information potentially conveyed in their odor.

Taken together, our results indicate that male mice modulate their scent-marking display according to their perceived mating opportunities and health and that females are better able to discriminate healthy versus infected males when the males are sexually stimulated and scent mark more. Our results are consistent with evidence that scent marking is costly (Gosling et al., 2000), and they suggest that mice modulate their marking to minimize their costs (because otherwise males should always mark at high frequency). Mice may modulate their scent marking to reduce their risk of predation (Kavaliers et al., 2001; Roberts et al., 2001), though

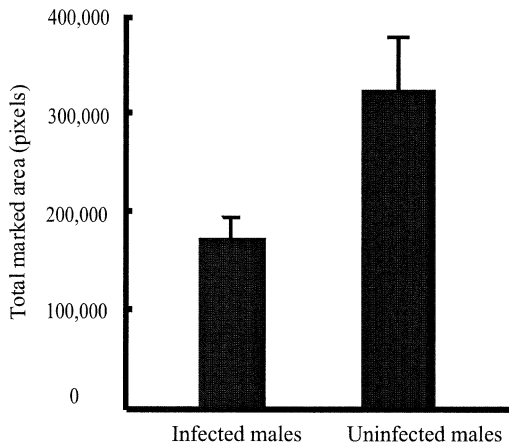


Figure 5

The area of paper that was scent marked by males that were experimentally infected versus sham-infected controls ($n = 33$ males; mean \pm SE).

we know of no direct evidence that scent marking increases the risk of predation. It has been suggested that males modulate their scent marking to reduce negative impacts on their immunological resistance to pathogens (Penn and Potts, 1998). Males increase their testosterone concentration, as well as scent marking, when they are sexually stimulated (Macrides et al., 1975), and a previous study found that sexual stimulation reduces males' resistance to infection (Smith et al., 1996). Males reduce their testosterone during infection (Hillgarth and Wingfield, 1997), and males' sexual odorants are testosterone dependent (Ferkin et al., 1994; Jones and Nowell, 1974; Novotny et al., 1984). These findings help explain how infection reduces the attractiveness of males' odor, and they are consistent with the immunocompetence handicap hypothesis (Folstad and Karter, 1992). However, the effects of testosterone on immunity are still not understood (Klein, 2000). Male mice also reduce the production of MUPs during infection (Isserhoff et al., 1986), and this could explain how infection reduces the attractiveness of males' scent. Moreover, MUPs are complex molecules, and their production is probably energetically costly and may divert or tie up amino acids and proteins necessary for immune functions. It is still unclear, however, whether scent marking or other secondary sexual traits impose physiological trade-offs with immune resistance.

We thank our research assistants, A. Bilak, S. Chambers, B. Church, T. Noetzel, M. Perkins, and E. Rodriguez, for their hard work on this project, and E. McClelland and E. Enioutina for help and advice. We also thank A. Bourke, S. Koyama, M. Gosling, and two anonymous referees for useful suggestions, and F. Goller, D. Clayton, D. Feener, and N. Hillgarth for their input and support. C. Hormaeche kindly provided the *Salmonella* strains. This research was funded by teaching stipends to S.M.Z. from the University of Utah and by a grant to N. Hillgarth from the National Science Foundation (IBN-9817008 A000).

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