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Genetic resistance to infection influences a male's sexual attractiveness and modulation of testosterone

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Abstract

Females may be attracted to males genetically resistant to infectious diseases, and one potential mechanism for this mating bias is that such males may be better able to maintain high testosterone. To test these two hypotheses, we collected scent-marks from male house mice (*Mus domesticus*) genetically resistant and susceptible to *Salmonella* due to a single locus (Nramp 1, also known as Slc11a1). We tested whether females are more attracted to the scent-marks of resistant males, and whether such males are better able to maintain testosterone concentrations during an experimental *Salmonella* infection. We found that females preferred the scent-marks of genetically resistant males compared to susceptible ones; but they showed no preferences 5 d after males were infected. As predicted, genetically resistant males maintained their testosterone concentrations during the experimental infection, whereas susceptible males showed a significant decline 14 d after inoculation. These differences in the males' ability to modulate testosterone, however, do not explain females' attraction to resistant males. Thus, our results indicate that females sometimes prefer males genetically resistant to infection, and they provide the first evidence that males modulate their testosterone depending upon their genetic resistance to infection; however, we found no evidence to link these two findings.

Keywords: Sexual selection; Hamilton–Zuk hypothesis; Immunocompetence handicap hypothesis; Testosterone; Mammals; House mice; *Mus*; *Salmonella*; Nramp; Scent-marking

1. Introduction

It is often suggested that females prefer males that are genetically resistant to infectious diseases (Hamilton and Zuk, 1982), but direct tests of this idea are lacking. Females prefer males with exaggerated secondary sexual traits, and such traits often indicate a male's parasite load or immunocompetence (Hamilton and Poulin, 1997; Møller et al., 1999), but it is unclear whether they reveal genetic resistance to infectious diseases. Most work on parasite-mediated sexual selection has addressed the mechanisms through which secondary sexual traits indicate parasite load or immunocompetence (Hillgarth et al., 1997). The leading idea proposes that genetically susceptible males cannot afford to maintain high concentrations of sex hormones, such as testosterone, necessary for the development and expression of secondary sexual traits due to their immunosuppressive properties (the immunocompetence handicap hypothesis) (Folstad and Karter, 1992). For example, testosterone controls the production of pheromones and scent-marking in male mice (Jemiolo et al., 1992; Novotny et al., 1990; Sam et al., 2001); however, it also inhibits T- and B-cell production, nitric oxide defenses, activates suppressor T cells, and subsequently reduces resistance to pathogens and parasites (Friedl et al., 2000;

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Grossman, 1984, 1985; Mossmann et al., 1997; Tanriverdi et al., 2003). These immunosuppressive effects are not necessarily maladaptive, as testosterone could function to allocate energy and resources between the competing demands of immunity versus reproduction (Wedekind and Folstad, 1994). This functional hypothesis could explain why T-lymphocytes and macrophages have androgen receptors (Benten et al., 2002a,b), and why testosterone acts on the androgen receptors of T lymphocytes to control production of interleukin-10, a cytokine that down-regulates a variety of antiviral responses, including antigen presentation (Liva and Voskuhl, 2001). Our goal in this study was to test whether females prefer males genetically resistant to infectious diseases compared to susceptible ones, and whether genetically resistant males are better able to maintain their testosterone concentrations during infection (the central assumptions of the Hamilton–Zuk and immunocompetence handicap hypotheses, respectively).

The evidence for the immunocompetence handicap hypothesis is mixed (Muehlenbein and Bribiescas, 2005; Roberts et al., 2004); however, conclusions are impossible due to several methodological problems. First, most studies have been conducted with birds, although their secondary sexual traits are not usually testosterone-dependant (Owens and Short, 1996). Second, many studies have manipulated testosterone, but the subsequent immunosuppressive effects might have been an artifact from using high (pharmacological) dosages or disrupting normal fluctuations (Hillgarth and Wingfield, 1997). For these reasons, we use mice to study how normal variations in testosterone affect resistance to pathogens (Zala et al., submitted for publication), and here we examined how males modulate their testosterone during infection. Third, most studies use antibody responses to antigens or other indirect immunocompetence assays to measure resistance to infectious diseases, but they assume that stronger responses are better and ignore immunopathology (Penn and Potts, 1998). Therefore, we examined how mice resolve and cope with an actual infection, using an avirulent strain of *Salmonella enterica* (serovar Typhimurium). Finally, studies are needed that manipulate *genetic resistance* to infection and examine the subsequent effects on males' secondary sexual traits and attractiveness to females (Kurtz and Sauer, 1999). We manipulated genetic immune resistance by using two congenic mouse strains, one that is resistant ('knock-in') and the parental strain which is susceptible to *Salmonella* and a variety of other pathogens (Vassiloyanakopoulos et al., 1998).

The scent-marks and other chemical signals that male mammals produce are functionally analogous to the colourful displays of birds and fish (Penn and Potts, 1998). Male mice increase their scent-marking courtship when they encounter novel females, which makes their scent more attractive to females (Zala et al., 2004). Females are less attracted to the scent of males during *Salmonella* infection (Zala et al., 2004) and other infectious agents (Kavaliers and Colwell, 1995; Penn et al., 1998). It is unclear how infection reduces the attractiveness of a male's scent, but this effect may be due to reductions in testosterone that occur during infection (Hillgarth and Wingfield, 1997; Klein and Nelson, 1998; Kong and Edmonds, 2002; Soudan et al., 1992; Spratt, 2001; Spratt et al., 1993; Willis and Poulin, 2000) or immune activation (Weil et al., 2006). Infected (and genetically susceptible) males may down-regulate the production of major urinary proteins (MUPs) during infection, and some evidence supports this idea (Isserhoff et al., 1986; Litvinova et al., 2005). No study to our knowledge, however, has tested whether females are more attracted to males that are genetically resistant to infection, or whether males modulate their testosterone according to their genetic resistance.

In this study, we found that females were more attracted to the scent-marks of genetically resistant compared to susceptible males before infection, but surprisingly, this preference was abolished during the experimental infection. We also found that genetically resistant males maintained testosterone during infection, whereas susceptible mice significantly reduced testosterone 2 weeks after *Salmonella* inoculation.

2. Materials and methods

2.1. Animals

We used 27 males from two congenic mouse strains, the parental strain genetically susceptible to *Salmonella* due to a single point mutation (11 BALB/c mice, which are Nramp−), and the resistant strain (16 BALB/c.D2 mice), which are Nramp+ knock-ins (Vassiloyanakopoulos et al., 1998). Nramp (natural resistance-associated-macrophage protein), also known as "Slc11a1" (solute carrier family 11 member 1) and previously known as "Ity/Lsh/Bcg," is the most important locus known for controlling resistance to *Salmonella*, and also affects resistance to many other infectious agents (Medina and North, 1998; Sebastiani et al., 1998). It encodes a membrane ion-transport protein exclusively expressed in the phagolysomes of macrophages where it restricts intracellular microbial growth by removing iron, manganese, and other divalent cations (Cannone-Hergaux et al., 1999; Ables et al., 2001). Our colony founders were obtained from different sources, and so we bred a new generation to control for potential confounding differences caused by colony conditions and age. At ca. 2 months of age, all the male mice were housed singly in cages ($30 \times 19 \times 13$ cm) containing pine bedding and paper towels for environmental enrichment. The mice were provided water and food (Harlan Teklad Rodent Chew) *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 the experiment were closely age-matched (usually born on the same day). For odor preference assays we used 22 virgin, estrous females of an outbred laboratory strain of mice (Swiss Webster) as smellers. We used an outbred rather than an inbred strain so that our results would be more general, and also because these mice can distinguish the odor of infected versus uninfected males (Zala et al., 2004). Females were kept under a 14:10 light:dark cycle, but otherwise under the same conditions as the males. All the animal experiments were conducted at the University of Utah, and were approved by the local Institutional Animal Care and Use Committee.

2.2. Experimental infection

We used an avirulent strain of *S. enterica* (serovar Typhimurium, 628 strain) (Hormaeche et al., 1985), which invades intestinal mucosa and replicates within host macrophages. We cultured bacteria in 20 ml of heart-

brain infusion at 37 °C for 12 h while shaking, diluted the overnight solution with sterile phosphate buffer solution to the desired concentration (1.5×10^3 bacteria per mouse, which takes at least 33 d to clear in BALB/c mice, unpublished data), and verified the concentration of viable bacteria by quantitative plate counts. All the male mice were infected intraperitoneally and bacterial loads were measured 15 d post inoculation. Pathogen loads were determined by sacrificing the mice (with CO_2), removing and homogenizing their spleens, serially diluting the homogenate on sterile selective agar plates, and counting the number of colony forming units per ml of homogenate (cfu/ml), using the mean of two replicates plates per mouse, after overnight incubation (37 °C). One BALB/c.D2 mouse had to be euthanized 2 d post inoculation as it was in poor condition.

2.3. Scent-mark collections

To compare the sexual attractiveness of males, we collected and analyzed scent-marks before and during infection. We collected scent-marks 18, 21, and 27 d after the males were singly caged (before infection) and also 5 and 9 d after inoculation (Fig. 1). Scent-marks were collected overnight on 7.5×7.5 cm filter papers. During collection, males were sexually stimulated with female urine (10 μl urine placed on a 4 cm^2 filter paper), using urine from different females for each collection, to activate courtship scent-marking (Zala et al., 2004). The scent-marked papers were stored at −70 ° C and we quantified the scent-marking patterns using a fluorescence scanner (Storm®), and a digital imaging program (AlphaEase™ 5.0) to count the number of marks and the total area marked (measured in pixels per defined area) (Zala et al., 2004).

2.4. Odor preference assays

We used a simultaneous choice assay to test the odor preferences of 22 females for resistant versus susceptible males (11 females for the scent-marks collected before infection, and another 11 for scent collected during infection). Odor assays were conducted under red light to simulate dusk, when the mice are most active (females light cycle was adjusted accordingly) and each female was test just once. The experimental apparatus was comprised of two acrylic cages, one 'start' and one 'test' chamber, with an acrylic cover to contain volatile odors, and connected by a plastic tube (Zala et al., 2004). Both chambers contained two smaller plastic 'hiding boxes,' and scent-markings were randomly presented in the hiding boxes of the test chamber. We began each trial by opening a remote-controlled door that allowed the female to enter the test chamber, and once the female entered the chamber (usually within 1–3 min), we closed a second remote-controlled door, and observed her behavior in the test chamber *via* a video camera and monitor. Observers naïve to the experimental design and identity of test males 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 amount of time she spent inside each box during the 7 min trial. Any bias that the females displayed towards the boxes was considered to be a preference. After each trial, the apparatus was washed with water and ethanol to remove residual odor traces.

2.5. Blood collections

To measure hormone concentrations, we collected blood from males four times, twice before and twice during infection. Blood was first collected 1 month after housing the males alone, and again 1 week later. One week after the second blood sample, we infected the mice, and 7 and 14 d post inoculation we collected additional blood samples (Fig. 1). The day after the final blood collection, mice were sacrificed to measure pathogen loads. Blood sampling was performed between 14:00 and 16:00 h to control for daily hormonal fluctuations. To collect blood, we placed the mice under a heat lamp for a few minutes to induce vasodilatation, and then immediately moved them to a specially designed mouse restrainer where we disinfected the tail and performed a superficial incision on the tail vein. Once we collected 100–200 μl blood, we compressed the tail to stop bleeding.

2.6. Radioimmunoassay (RIA) procedures

Following collection, whole blood samples were maintained at 4 °C for 24 h. Samples were then centrifuged at room temperature for 10–15 min at 12.000 rpm, and the supernatant was collected and frozen at −70 °C. Frozen samples were packed on dry ice and shipped over night to the Ohio State University (Columbus, OH), where they were assayed for testosterone and corticosterone. Serum testosterone concentrations were determined in duplicate using an ICN ^{125}I ImmuChem Coated Tube kit (Costa Mesa, CA), and the high and low limits of detectibility of the assay were 20 and 0.2 ng/ml, respectively. Serum corticosterone concentrations were determined in duplicate using an ICN ^{125}I Double Antibody kit for mice (Costa Mesa, CA), and the high and low limits of detectibility of the assay were 1000 and 5 ng/ml, respectively. These assays are highly specific, cross-reacting with less than 1% of other hormones. Average assay sensitivities for corticosterone and testosterone were 5.2 ng/ml and 0.18 ng/ml, respectively. The inter- and intra-assay coefficients of variation were <10% in all cases. 16 testosterone samples exceeded the upper detection limit of the standard curve. We analyzed the data by excluding these non-detectable samples, which reduces the sample size, and also by including these samples by assigning the highest (20 ng/ml) value of the measurable range.

2.7. Statistical analyses

We used JMP (SAS Institute Inc., version 5.0.1.2) and the statistical package R (R Development Core Team, 2007) to analyze the data. We used parametric tests only after checking the assumptions, and transforming non-normal data. The testosterone data that included the 16 non-detectable samples were not normally distributed and could not be normalized. Omitting these 16 non-detectable samples outside the standard curve allowed normalization using a logarithmic transformation. All the normal distributed hormonal data were analyzed with an analysis of variance (ANOVA) with repeated measurements in a linear mixed effect (lme) model with "time" (blood collection before versus after inoculation) and "strain" as fixed factors and animal identification as random factor using the library nlme (Pinheiro et al., 2007) within the statistical package R (R Development Core

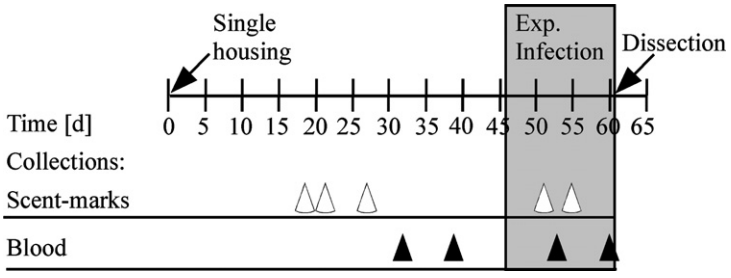

Fig. 1. Timeline of the experimental procedures.

Team, 2007). We used the interaction between "time" and "strain" to test whether testosterone is lowered more in the susceptible than the resistant strain during infection. Because there are no non-parametric models for testing such interactions we used a Wilcoxon ranked-sum test to analyze the non-normally distributed testosterone data. We utilized 2-tailed tests, or when we had *a priori* prediction, we used directed tests (Rice and Gaines, 1994) (which are more conservative than 1-tailed tests, and allow the analysis of results that go in the opposite of the predicted direction). Results are reported as mean ± 1 standard deviation unless stated otherwise, and differences were considered statistically significant when $p \leqslant 0.05$.

3. Results

We first confirmed that our Nramp+ mice were more resistant to *Salmonella* infection than the Nramp− strain (as a positive control). In the first experiment, we infected the Nramp+ mice we obtained from another laboratory and found that they had significantly lower *Salmonella* loads compared to the Nramp− controls reared in our laboratory (*t*-test: $N = 19$, $df = 17$, $t = -2.5$, $p_{dir} = 0.014$). In the second experiment, in which we reared the mice in the same laboratory (and used for the experiments below), we found a trend in the same direction (*t*-test: $N = 26$, $df = 24$, $t = -1.3$, $p_{dir} = 0.132$), and the difference between these strains is significant when we pool these experiments (*t*-test: $N = 45$, $df = 43$, $t = -2.1$, $p_{dir} = 0.024$).

We tested the females' preferences for the scent-marks of Nramp+ versus Nramp− males before and during *Salmonella* infection, and we found that females showed a significant preference for resistant males, but not 5 d after we infected the males (Fig. 2). Before infection, females spent more time in the hiding boxes containing the scent of the resistant compared to the susceptible males (Wilcoxon signed-ranks test: $N = 11$, WSR = -23, $p = 0.04$, Fig. 2a). Females' initial preference (binomial test: $N = 11$, $p = 1$) and number of visits to the hiding boxes (paired *t*-test: $N = 11$, $df = 10$, $t = -1.2$, $p = 0.28$) did not differ significantly. Five days after the experimental inoculation, however, females showed no difference in time spent in the hiding boxes (Wilcoxon signed-ranks test: $N = 11$, WSR = 1, $p = 0.97$, Fig. 2b), initial preference (binomial test: $N = 11$, $p = 1$), or number of visits (paired *t*-test: $N = 11$, $df = 10$, $t = 0.4$, $p = 0.71$). We found no difference in the amount of scent-marks between the resistant and the susceptible males either before (*t*-test: number of spots, $N = 27$, $df = 25$, $t = -0.4$, $p = 0.69$; area, $N = 27$, $df = 25$, $t = -0.1$, $p = 0.92$) or 9 d after inoculation (*t*-test: number of spots, $N = 26$, $df = 24$, $t = -0.5$, $p = 0.63$; Wilcoxon ranked-sum test: area, $N = 26$, $Z = 0.83$, $p = 0.41$).

We also compared how the males modulated their testosterone concentrations during the course of infection. We found that the males' testosterone concentrations did not differ before the experimental infection, but after inoculation, the resistant males maintained higher testosterone concentrations whereas the susceptible males reduced theirs (Fig. 3). When we analyzed the data excluding the 16 non-detectable data points, we found a significant interaction between "time" (before and post inoculation) and "strain," both when we included only the second week after infection ($df = 1,16$; $F = 5.996$; $p_{dir} = 0.016$) or both weeks ($df = 2,34$; $F = 2.827$; $p_{dir} = 0.046$). The main factors "time" and "strain" were not significant in the models. When we analyzed the testosterone data and included the 16 non-detectable samples, the results remained the same. The testosterone concentrations of the two strains were not significantly different until 14 d after the experimental inoculation (2 weeks before inoculation: $N = 27$, $Z = -1.2$, $p = 0.23$; 1 week before inoculation: $N = 27$, $Z = -0.4$, $p = 0.71$; 1 week after inoculation: $N = 25$, $Z = -0.8$, $p = 0.44$ and 2 weeks after inoculation $N = 26$, $Z = -2.7$, $p_{dir} = 0.005$). In comparison, the resistant and susceptible mice did not differ in modulating their corticosterone during the experimental infection (model with only second week post

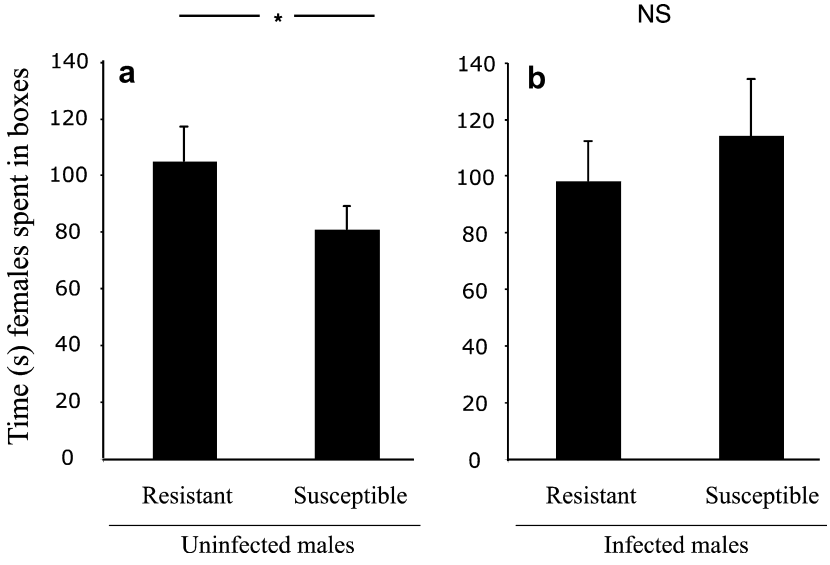

Fig. 2. Time (mean ± 1 standard error) female mice spent in the hiding box of resistant and susceptible males, both before (a), and during experimental infection (b). (NS, non significant; *significant difference at $p < 0.05$.)

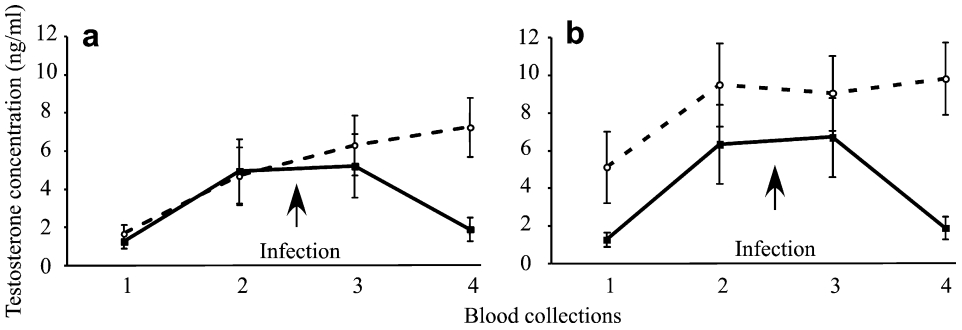

Fig. 3. Testosterone concentrations (ng/ml) of males collected over five weeks (mean ± 1 standard error) showing (a) only the data within the standard curve, and (b) all the data (with adjusted values). Samples were collected 2- and 1-week before infection, and then one and two weeks after experimental inoculation. Open circles with a dashed line represent resistant mice and filled squares with solid line show susceptible mice.

inoculation: $df = 2,44$; $F = 0.27$; $p = 0.76$; model including both weeks: $df = 3,68$; $F = 1.26$; $p = 0.295$).

4. Discussion

The females were significantly more attracted to the scent-marks of males carrying the resistant Nramp gene compared to susceptible ones prior to our experimental *Salmonella* infection. This finding supports the idea that females can recognize and prefer to mate with genetically resistant males, although actual mating preferences still need to be tested. We found no difference in the amount of scent-marking between resistant and susceptible males, either before or during infection, which suggests that females used qualitative differences in the male's scent rather than the amount of scent-marking. We also found that genetically resistant males maintained testosterone concentrations during the experimental infection, whereas susceptible males showed a significant decline 2 weeks after *Salmonella* inoculation (this was true whether we include the non-detectable samples or not). Interestingly, this decline roughly corresponds to the time that the immune system begins to resolve *Salmonella* infection in these mice (O'Callaghan et al., 1988). This result directly supports the idea that genetically resistant males are better able to maintain testosterone concentrations during an active infection compared to susceptible males, which is a central assumption of the immunocompetence handicap hypothesis. It also suggests that testosterone reduction during infection is a functional response to avoid immunosuppressive effects, rather than simply a pathological side-effect, as often assumed (which is concerning since testosterone replacement is often prescribed to HIV-infected patient; Spratt, 2001). Using a non-replicating pathogen or other vaccine would provide a stronger test of this idea.

It is unclear how infection causes a reduction of testosterone during infection, or why this reduction occurred only in the genetically susceptible mice, though we suggest a potential mechanism. Mice elevate an array of proinflammatory cytokines during *Salmonella* infection, which play pivotal roles in controlling bacterial growth, and at least two cytokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) are also potent modulators of testicular Leydig cell steroidogenesis, causing a decrease in testosterone concentrations (Bornstein et al., 2004; Hedger and Meinhardt, 2003; Hong et al., 2004; Watanobe and Hayakawa, 2003). One study found that Nramp susceptible mice are capable of mounting normal cytokine responses during *Salmonella* infection (their susceptibility is not due to defects in cytokine responses), and when compared to congenic controls during infection, they had up to fourfold greater elevations in the expression of mRNA levels of several cytokines, including TNF-α even at the same bacterial counts—and this difference became more apparent during the course of infection (Eckmann et al., 1996). Thus, future studies could examine whether testosterone concentration reduction in susceptible mice is due to their having greater (or prolonged) elevations of IL-1, TNF-α or other cytokines during infection compared to resistant mice.

Contrary to our expectation, however, we found no evidence that females' preferences for genetically resistant males were due to the males' testosterone concentrations, as there was no significant difference in the concentrations before the experimental infection, when females showed a preference. We expected to find a difference in testosterone concentration between these strains even before the experimental infection because Nramp affects resistance to a wide range of infectious agents (Zhang et al., 2000), which may include those found in conventional colony conditions, such as *Staphylococcus aureus*, *S. epidermidis*, and *Streptococci*. One possibility is that our hormone assay was not sufficiently sensitive to detect small differences that may have occurred before the experimental *Salmonella* infection. Indeed, when we include the 16 samples in our analysis that were beyond the range of our standard curve, the results suggest that testosterone is consistently higher in the resistant mice (Fig. 3b). Another possibility is that genetically resistant males are able to maintain higher testosterone concentrations than susceptible males at critical periods of development or small differences have cumulative effects over their lifetime (Penn and Potts, 1998). Of course, it is possible that testosterone plays no role in influencing how females recognize healthy, genetically resistant males, though this seems unlikely given that mice downregulate testosterone and the expression of MUPs during infection (Isserhoff et al., 1986), and immune activation

reduces a male's concentration of urinary proteins (Litvinova et al., 2005).

Also contrary to our expectation, the females' preference for genetically resistant males was not more pronounced during the experimental *Salmonella* infection, and was actually abolished. There are several possible reasons for this result. First, both the resistant and susceptible males were infected in our study and perhaps females are not attracted to any infected males (nearly all mice in our study, both susceptible and resistant, remained infected at necropsy—15 d post inoculation—and must have had a higher bacterial load at the time of scent collection, 5 d post inoculation). Second, collecting blood was probably stressful on the males, which may have reduced their attractiveness to females or activated fearful responses in females. Another possible explanation is that we only tested female preferences 5 d after the males were inoculated, and testosterone concentrations had not yet declined significantly in the susceptible males. Unfortunately, we did not test attraction to scent-marks when the susceptible males' testosterone became significantly lower, 2 weeks after experimental inoculation. Future work on this problem should consider examining larger (quantitative) genetic effects on resistance to infection (as the Nramp mutant is merely a single point substitution) and also test these hypotheses in wild mice under more natural conditions.

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