Behav Ecol Sociobiol (2008) 62:895–900
DOI 10.1007/s00265-007-0513-0

ORIGINAL PAPER

Exposing males to female scent increases the cost of controlling *Salmonella* infection in wild house mice

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Received: 27 March 2007 / Revised: 10 October 2007 / Accepted: 20 October 2007 / Published online: 23 November 2007

Abstract Secondary sexual characters often provide indicators of a male's resistance to infectious diseases to rivals and potential mates, but it is unclear why. It is often suggested that males honestly signal their health due to energetic and other physiological trade-offs between investing into secondary sexual traits vs resistance to infectious diseases. Our aim was to determine whether such a trade-off exists using wild-derived male house mice (*Mus domesticus*). We exposed male mice to female scent, a manipulation that induces elevations in testosterone concentration and the expression of a variety of testosterone-mediated secondary sexual traits, and tested whether this sexual stimulation impaired the males' ability to resolve or cope with an experimental infection (*Salmonella enterica*). We kept the males on a controlled diet to prevent them from compensating by eating more food. We found that sexually stimulated males were able to control bacterial growth as effectively as sham-stimulated controls; however, to do so, they lost more body mass during infection compared to the controls. In contrast, we found no evidence that sexual stimulation reduced the body mass of uninfected male mice. These results indicate that males' responses to female odor are not immunosuppressive per se, yet they increase the energetic costs of controlling infection. Our findings support the idea that there is a physiological trade-off between secondary sexual signaling vs resistance to infectious diseases and suggest that studies using only immunocompetence assays might fail to detect such energetic trade-offs.

Keywords Ecological immunity · Testosterone · *Salmonella* · House mice · Sexual selection · Immunocompetence handicap hypothesis

Communicated by J. Wilkinson

We dedicate this paper to the late Professor Chris Barnard who conducted pioneering research on this topic.

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Introduction

Exaggerated secondary sexual traits, such as the colorful plumage found in males of many species, often provide honest indicators of their health and resistance to infectious diseases to rivals and potential mates (Hamilton and Zuk 1982; Hamilton and Poulin 1997; Møller et al. 1999). Several hypotheses have been proposed to explain how these traits reflect health and disease resistance. The handicap hypothesis suggests that elaborate secondary sexual traits provide reliable indicators of a male's condition or quality because only high-quality males can afford the costs of these traits (Zahavi 1975). Handicap models often rely on assumptions about the energetic costs of signals, whereas Folstad and Karter (1992) argued that energetic costs are not sufficient and instead proposed that honest signaling is due to a physiological trade-off that arises because testosterone (or other biochemical self-regulated substances) has a dual effect, enhancing secondary sexual traits but also impairing immune responses (immunocompetence handicap hypothesis). To explain this

dual effect, it was suggested that testosterone adaptively redistributes energy, metabolites, and other resources between sexual ornaments vs immune function due to underlying trade-offs between these traits (Wedekind and Folstad 1994). Other simpler hypotheses have been suggested to explain honest signaling that do not require the dual effect of testosterone, and instead rely on trade-offs due to energetic or other limiting resources (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). The goal of this study was to examine the assumption that there are physiological trade-offs between secondary sexual traits and immune defenses using wild-derived house mice (*Mus domesticus*).

Although male house mice are visually drab, they display a variety of complex secondary sexual traits that are sexually dimorphic and testosterone-dependent, including ultrasonic courtship vocalizations (Nunez and Tan 1984), scent-marking behavior (Kimura and Hagiwara 1985), sexual pheromones (Novotny et al. 1990), and pheromone-binding major urinary proteins (Hastie et al. 1979). Males mark more conspicuously when they are socially dominant (Desjardins et al. 1973) or presented with mating opportunities (Zala et al. 2004). Females are attracted to males' scent marks (Hurst 1990), especially from dominant (Drickamer 1992) and more competitive males (Rich and Hurst 1998; Gosling and Roberts 2001). Females also prefer the scent of uninfected compared to infected males (Kavaliers and Colwell 1995; Penn and Potts 1998). In a previous study, for example, we found that males reduced their scent-marking when infected with an avirulent strain of *Salmonella*, and females were less attracted to their scent marks compared to sham-infected males (Zala et al. 2004). Scent-marking (Gosling et al. 2000) and immune responses (Demas et al. 1997; Lochmiller and Deerenberg 2000) are both energetically costly, but it is unknown whether these traits impose physiological trade-offs for each other. Studies with other species indicate that reproduction can adversely affect immune function (Deerenberg et al. 1997; Nordling et al. 1998; Ilmonen et al. 2000), yet it is unclear whether investing into secondary sexual traits imposes a negative trade-off with immune functions or vice versa.

The evidence for the immunocompetence handicap hypothesis is mixed (Roberts et al. 2004; Muehlenbein and Bribiescas 2005), and conclusions are difficult mainly due to several methodological problems. First, experimentally increasing testosterone in mice, for example, reduces immune functions (Grossman 1984; Grossman 1985) and resistance to parasites (Wunderlich et al. 1988, 1991; Mossmann et al. 1997); however, these effects might be a pathological side effect of manipulating this hormone above normal ranges (or removing its normal fluctuations) (Hillgarth et al. 1997). Therefore, studies are needed that manipulate testosterone and investment into testosterone-mediated sexual traits within normal ranges. It is interesting to note that exposing male mice to novel females or their urinary odor induces an increase in testosterone concentration (Macrides et al. 1975; Maruniak and Bronson 1976; Batty 1978a, b; Tran, Zala and Potts, unpublished data), and a variety of testosterone-mediated responses, including ultrasonic courtship vocalizations (Whitney et al. 1974), copulatory behavior (James and Nyby 2002), and preputial gland development and sperm density (Koyama and Kamimura 2000). Previously, we found that sexual stimulation increases the scent-marking behavior in male mice and the attractiveness of their scent marks to females (Zala et al. 2004). Also, male mice modulate their testosterone concentration during *Salmonella* infection depending upon their genetic resistance to pathogens (Zala et al. 2007). In the present study, we aimed to test whether sexual stimulation reduces males' resistance to infectious disease.

Second, behavioral ecologists have generally relied on immunocompetence assays, such as antibody responses to sheep red blood cells or other foreign antigens, but these tests do not necessarily indicate resistance to infectious agents (Penn and Potts 1998; Adamo 2004). Moreover, these studies often assume that stronger immunological responses are better, ignoring the damaging effects of the immune system (immunopathology) (Penn and Potts 1998). Therefore, studies are needed that measure males' resistance to actual infectious agents because disease resistance better reflects how individuals balance pathogen clearance with minimizing immunopathology ("optimal immunocompetence") (Penn and Potts 1998; Viney et al. 2005). Previous findings report mixed evidence for the idea that male laboratory mice face a trade-off between responding to female odors and an immune challenge (Smith et al. 1996; Barnard et al. 1997a, b); however, the significant findings only assessed antibody responses to sheep red blood cells rather than resistance to infectious agents. Therefore, in the present study, we examined the pathogen clearance and disease resistance (body mass and survival) of mice when challenged with an actual mouse pathogen, *Salmonella enterica*.

Third, testing for physiological trade-offs due to energetic and other resource limitations requires controlling food intake because infected individuals might compensate by eating more food (Lochmiller and Deerenberg 2000; Moret and Schmid-Hempel 2000). Therefore, we carefully controlled the food intake of the mice in our experiments.

Material and methods

Subjects and housing We used 40 wild-derived sexually mature male house mice (*M. domesticus*) maintained in an

outbred colony (Meagher et al. 2000), and the odor donors for sexual stimulation were adult females (Swiss Webster). All the males were singly housed in acrylic cages (30×19×13 cm) containing pine bedding and paper towels for environmental enrichment. Females were provided with water and food ad libitum. Males were provided with water ad libitum, but during the infection they were kept on a restricted food diet (3.0–3.2 g Harlan Teklad Rodent Chow per day) until dissection. We controlled food intake because our goal was to test whether there is an energetic trade-off between the males' ability to clear an infection and to produce attractive odors, and therefore, we needed to eliminate the possibility that infected males might compensate simply by eating more food (Moret and Schmid-Hempel 2000). All the mice were 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.

Sexual stimulation To induce elevations in testosterone concentrations (Macrides et al. 1975; Tran, Zala and Potts, unpublished data) and a variety of testosterone-dependent secondary sexual traits, including scent-marking (Zala et al. 2004), we exposed males to the odor of novel females. We placed one filter paper (2×2 cm) containing 10 μl of female urine (or water for the controls) into the males' cages every day (five times per week) (Zala et al. 2004), starting at the day of the infection. All the males within each experiment were offered the treatments at the same time. The urine was previously collected from 32 adult female Swiss Webster mice by bladder palpitation. We pooled the urine samples over 7–20 days of collection for each female to control for variation in odor due to estrous state and stored the urine at −70°C. We alternated the stimulus urine from different females every time because males increase their testosterone when exposed to novel females (Macrides et al. 1975). Using female odor as a stimulus, rather than the presence of females, controls for other potential confounding factors, such as energetic costs due to chasing females. To provide clear substrate for males to scent-mark, we placed sterile filter papers (7.5×7.5 cm) in the males' cages every few days.

Disease resistance To measure resistance to infectious disease, we examined the males' ability to clear a bacterial infection and maintain body mass during infection. We used *S. enterica* (serovar Typhimurium, strain 628) (Hormaeche et al. 1985), which is avirulent in wild and outbred mouse strains (Penn and Zala, unpublished data). We cultured bacteria in 20 ml of heart–brain infusion at 37°C for 12 h while shaking and diluted the overnight solution with sterile phosphate buffer solution (PBS) to the desired concentration. We used this solution to infect 20 male mice intraperitoneally (inoculum dosage 1×10^4 cfu/mouse). Ten and 11 days after the infection, we assayed pathogen clearance by euthanizing the mice (with CO_2) and dissecting their spleen. We homogenized the spleens (in 1 ml of PBS) under sterile conditions. We performed serial dilutions of the spleen homogenates, incubated them overnight (37°C), and determined the pathogen loads by counting the number of colony forming units per milliliter of spleen homogenate on the plates (concentration of bacteria per spleen). We used the mean of two replicate plates per mouse. We assayed the ability of the mice to cope with *Salmonella* infection by monitoring changes in body mass, which indicates disease pathology (Penn et al. 2002). We then repeated this experiment with uninfected mice to determine the effect of sexual stimulation on body mass without infection. Twenty additional adult wild-derived male mice were sexually stimulated with female urine or with water (controls) and kept on the same food regime and conditions as described above. We monitored the body mass changes during 10 days (just as above) and continued for a total of 59 days (to investigate the effect of longer treatment).

Statistical analyses We used JMP (SAS Institute, version 5.0.1.2) to analyze the data. We used (two-sided) parametric tests only after checking for normality assumptions, and transforming nonnormal data. Changes in male body mass during the experiment were analyzed with an ANCOVA model using body mass at dissection (first experiment) or at 10 days (second experiment) as the dependent variable, the initial body mass as a covariate, and the treatment (female urine or water stimulation) as the factor in the analysis. The results are reported as mean±1 SD, unless stated otherwise.

Results

Sexual stimulation and infection At dissection, the mean bacterial load of the sexually stimulated males was not different from the controls ($1.0\times10^3\pm6.1\times10^2$ cfu/ml for the stimulated males compared to $3.1\times10^3\pm4.31\times10^3$ cfu/ml for the control males; *t*-test for unequal variances: $t_{(10.93)}=0.5$, $p=0.63$, Fig. 1). It is interesting to note that the sexually stimulated males had a significantly lower variation in bacterial load than the controls (Brown–Forsythe test: $F_{(1,17)}=7.13$, $p=0.016$, Fig. 1). During infection, sexually stimulated males lost significantly more body mass compared to the sham-stimulated males (Fig. 2a; dissection weights: stimulated=17.2±2 g vs controls=20.7±2 g; ANCOVA: $F_{(1,16)}=6.5$, $p=0.021$).

Sexual stimulation without infection The reductions in body mass of the sexually stimulated males may have occurred even without infection due to the energetic costs

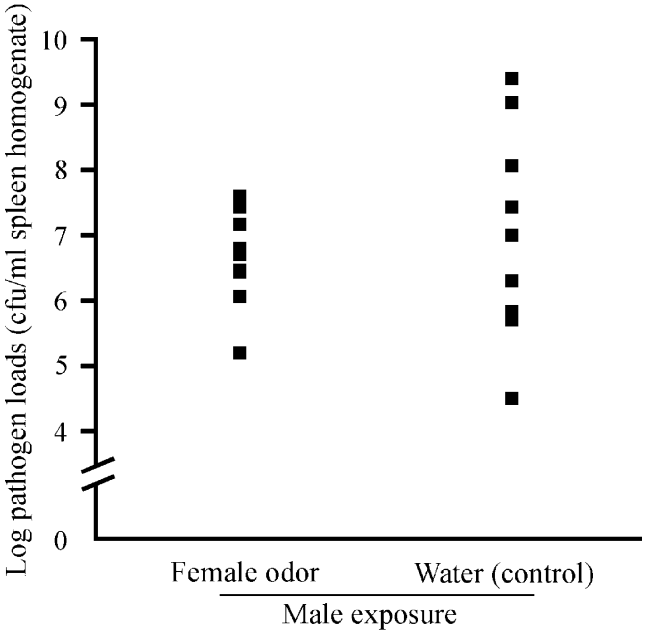

Fig. 1 Pathogen loads of sexually stimulated males compared to control stimulated males 10–11 days after experimental infection with *Salmonella* (cfu/ml spleen homogenate)

of this behavior; and to rule out this possibility, we repeated the experiment using uninfected mice. Sexual stimulation alone did not significantly change the male's body mass during the first 10 days (Fig. 2b; dissection weights: stimulated=17.8±2 g vs controls=18.2±2 g; ANCOVA: $F_{(1,17)}$=0.002, p=0.97) nor after 59 days of treatment (Fig. 2b; dissection weights: stimulated=18.9±2 g vs controls=19.5±1 g; ANCOVA: $F_{(1,17)}$=0.66, p=0.43).

Pooling both experiments We also analyzed both datasets together, using a two-way ANCOVA model with body mass at dissection (or 10 days) as the dependent variable, the initial body mass as a covariate, and treatment (female urine or water stimulation) and experiment (infection or not) as main factors. This analysis showed that treatment ($F_{(1,34)}$=6.4, p=0.016), experiment ($F_{(1,34)}$=6.3, p=0.017), and the interaction between the two main factors ($F_{(1,34)}$= 8.5, p=0.006) are all significant. The interaction is the result of interest, and it indicates that the negative effect of sexual stimulation on the males' body mass depended upon the males being infected.

Discussion

We found no evidence that sexual stimulation impaired the males' ability to clear *Salmonella* infection, contrary to our expectation, although we found significant variation in pathogen loads between the treatment and controls. This unexpected result suggests that sexual stimulation made the males' immune defenses more uniform (Fig. 1). As predicted, sexually stimulated males lost more body mass during infection compared to sham-stimulated controls. In our second experiment using uninfected males, we found no evidence that sexual stimulation affected males' body mass (even after we monitored their weight for an additional 49 days), which rules out the possibility that weight loss might be due to sexual stimulation alone. We compared our two experiments in a single analysis, and the significant interaction between stimulation treatment and infection again indicates that the effect of sexual stimulation on weight loss depended on infection. We conducted the second experiment at a later date, and therefore, the strongest conclusions can be drawn from comparisons of the treatment and controls within (rather than between) the two experiments (in separate analyses). This caveat is especially important given that we found differences in the males' weight loss between these two experiments (for unknown reasons, the stimulated and control males both

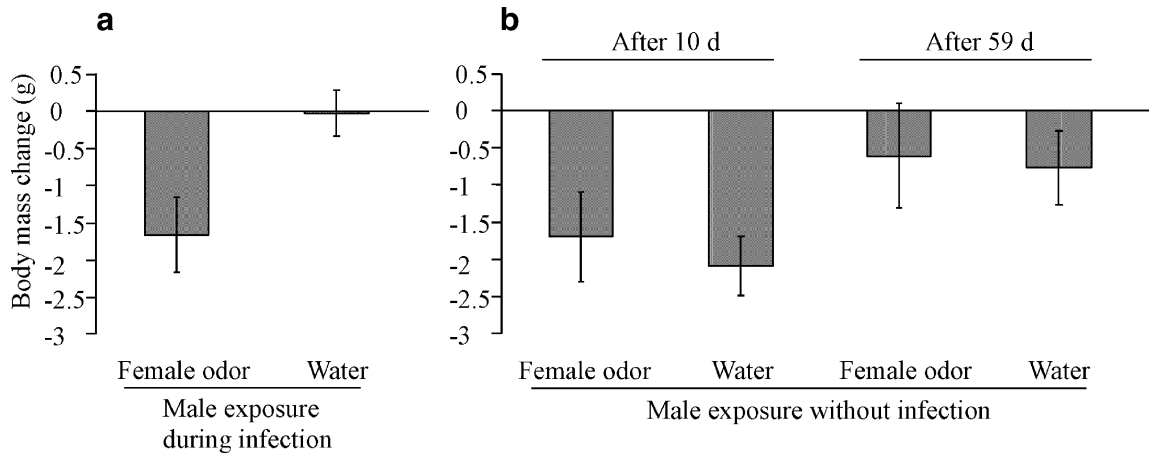

Fig. 2 Body mass changes in sexually stimulated males compared to control stimulated males during an experimental *Salmonella* infection (**a**) and without infection after 10 days and after 59 days of treatment (**b**). Shown are the means and standard errors

lost weight in the second experiment, particularly during the first 10 days). Our second experiment is an important control, especially because another study found that competitive countermarking by male laboratory mice induced by chronic exposure to male scent is energetically costly, as measured by growth rate (Gosling et al. 2000). Thus, our results do not support the hypothesis that normal elevations of testosterone are immunosuppressive per se, as often assumed (Folstad and Karter 1992) and reported in studies using immunocompetence assays (Barnard et al. 1997a, b), yet they indicate that sexual stimulation makes controlling bacterial growth more energetically costly. Our results help to explain why males often reduce testosterone concentrations during infection, and particularly when they are genetically susceptible to infection (Zala et al. 2007). They also help to explain why studies using immunocompetence assays often fail to find the expected results (Roberts et al. 2004; Muehlenbein and Bribiescas 2005): responding to foreign red blood cells or other antigens is probably not as energetically demanding as resisting infection with an actual pathogen.

The physiological mechanisms underlying the physiological trade-off between the males' responses to sexual stimulation and disease resistance are not entirely clear. Our results suggest an energetic trade-off, but another possible explanation for why sexual stimulation reduced the body mass of infected males is that the combination of these two treatments reduced the males' appetite. This possibility is unlikely, however, because the mice in all the groups ate all of their food almost without exception. Testosterone may have played a role mediating this trade-off because many studies, including our own, show that sexual stimulation triggers elevations in testosterone (Macrides et al. 1975; Maruniak and Bronson 1976; Batty 1978a, b; Tran, Zala and Potts, unpublished data) and a variety of testosterone-dependent responses, such as scent-marking (Zala et al. 2004). However, we did not directly manipulate or measure testosterone in this study (collecting blood from mice is very intrusive and stressful, and we did not want to jeopardize the goals of our experiment), and our finding could also be due to nontestosterone mediated mechanisms. Although it emphasizes the role of testosterone, the immunocompetence handicap hypothesis accommodates "any biochemical substance that is self-regulated and exerts the two-pronged effect of compromising the immune system and stimulating trait expression" (Folstad and Karter 1992). Sexual stimulation may also elevate the males' metabolic rate and tie up critical protein or other resources necessary for immunity. For example, male mice produce large quantities of major urinary proteins (MUPs) for scent-marking, which are probably energetically costly and may compete for proteins or metabolic pathways needed for disease resistance (Zala et al. 2004). A recent study with mice found that immune activation results in a reduction of urinary proteins, and testosterone administration abolished this effect (Litvinova et al. 2005), suggesting that males sacrifice the production of MUPs for immunity and that testosterone plays a role in this reallocation. Still, other studies report mixed evidence for testosterone modulating trade-offs between male responses to female odor and immunity (Smith et al. 1996; Barnard et al. 1997a, b).

In summary, previous studies have found that exposing male mice to the odor of females triggers elevations of testosterone concentrations and investment into testosterone-mediated secondary sexual traits; and in this study, we report that such sexual stimulation made controlling bacterial growth more energetically costly—even though it did not reduce the males' ability to resolve a *Salmonella* infection. Our findings suggest that investing into secondary sexual traits need not impact immunity per se (pathogen clearance) to impose physiological trade-offs on disease resistance. Our study provides evidence for a physiological trade-off between males' responses to mating opportunities and disease resistance; and it is the first evidence, to our knowledge, that this trade-off is mediated by energetic constraints rather than immunocompetence per se. Future work should address how males' responses to mating opportunities, including variation in testosterone concentration and testosterone-mediated traits, affect disease resistance, and whether providing males with an ad libitum diet eliminates energetic trade-offs.

Acknowledgements We thank our assistants B. Chan and J. Tanner for their help; W. Arnold, D. Clayton, D. Feener, F. Goller, N. Hillgarth, and P. Ilmonen for their input and support; R. Hengsberger for the assistance; and G. Wilkinson and two anonymous referees for the suggestions that improved the manuscript. We are grateful to the late C. Hormaeche for kindly providing us with the *Salmonella*. This research was funded by teaching stipends to S.M.Z. from the University of Utah, the Austrian Academy of Sciences, and a grant from the National Science Foundation (IBN-9817008 A000). All the experiments were conducted at the University of Utah, were approved by the local Institutional Animal Care and Use Committee, and comply with the laws of the country in which they were performed.

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