

FEMALE SCENT SIGNALS ENHANCES MALE RESISTANCE TO INFLUENZA

Ekaterina A. Litvinova¹, Elena P. Goncharova², Alla M. Zaidman³, Marina A. Zenkova²,
Mikhail P. Moshkin^{1,4}

¹Institute of Systematics and Ecology of Animals, SB RAS, Frunze 11, Novosibirsk,
630091, Russia.

²Institute of Chemical Biology and Fundamental Medicine, SB RAS, Lavrentyeva 8,
Novosibirsk, 630090, Russia.

³Novosibirsk Research Institute of Traumatology and Orthopaedics, Frunze 17,
Novosibirsk, 630091, Russia.

⁴Institute of Cytology and Genetics, SB RAS, Lavrentyeva 10, Novosibirsk, 630090,
Russia.

Summary paragraph

Scent of receptive females as signal to reproduction stimulate male mice to olfactory search of a potential breeding partner^{1,2}. This searching behavior is coupled with infection risk due to bacterial contamination of the fecal and urine scent marks⁴. The theoretical consideration of host evolution under inevitable parasitic pressures, including helminthes, bacteria, virus etc., predicts adaptations that help protect against parasites associated with breeding⁷. In this study, we propose that acceptance of female signals by male mice leads to adaptive redistribution of immune defense directed to protection against respiratory infection risks. Our results reveal migration of macrophages and neutrophils to upper airways upon exposure to female odor stimulus resulting in increased resistance to influenza virus in male mice. Contrary to widely accepted immunosuppressive function of female sexual signals, our data provide the first demonstration of the adaptive immunological response to female odor stimulus through induction of nonspecific immune response in upper airways.

The scent of female mice as a signal for reproduction stimulates male mice to search for a breeding partner¹⁻³. In turn, searching behavior increases risk of respiratory infections in male mice due to their sniffing of the contaminated female fecal and urinary marks, which harbor many infection agents⁴⁻⁶. The theoretical consideration of host evolution under inevitable parasitic pressures, including helminthes, bacteria, virus etc., predicts adaptations that help protect against parasites associated with breeding⁷. We hypothesized that the scent of female soiled bedding as most naturalistic sexual signal⁸⁻¹⁰ induces integrative neuroendocrine response, which activated migration of immunocompetent cells according to breeding related change of the infection risks. Here we studied leukocyte trafficking into upper air-ways and resistance to respiratory infection (influenza as an example) in male mice kept with or without female soiled bedding during 1-3 weeks. In all experiments BALB/c male and females, 12-16 weeks old, were maintained in unisex groups of 4-5 animals under standard temperature, light-dark period (12:12 h) and food and water *ad libitum*.

Resistance to influenza. Mature BALB/c males were kept in two odor environments: with (n=41) or without the exposure (n=41) to the female soiled bedding as most naturalistic sexual signals in the mouse. One week later, they received an intranasal application (while under ether anesthesia) of the mice adapted influenza virus A/WSN/33 (H1N1) in 50 μ l phosphate-buffered saline (PBS). We used 5 serially diluted doses that contain from 2.5×10^1 to 2.5×10^5 focus-forming units (FFU). The mice were observed daily for the following 3 weeks. The total mortality rate was lower in the males exposed to the female bedding (8 from 41), in comparison with male mice not exposed (19 from 41 mice, $\chi^2 = 5.52$, $P = 0.019$). The males exposed to the female bedding had a higher LD₅₀ than the males isolated from females (Fig. 1).

Male mice were challenged with mouse-adapted influenza virus A/WSN/33 (H1N1) by intranasal inoculation. The total mortality rate was lower in the males exposed to the female bedding (8 from 41), in comparison with not exposed male mice (19 from 41 mice, $\chi^2 = 5.52$, $P =$

0.019). The males exposed to the female bedding had a higher LD₅₀ than the males isolated from females (Fig. 1).

Lung histology and broncho-alveolar lavage (BAL). A possible mechanism of scent induced resistance to respiratory infection may be the migration of immune cells to body compartments where they are likely to be more effective, for example to upper airways. Therefore we have made histological preparations of lung tissue harvested from the additional groups of non-infected male mice kept either exposed (n=3) or non-exposed (n=3) to female bedding. We found a significantly increased number of leukocytes in the perivenous and peribronchial areas in male mice that were exposed to the female bedding (Fig. 2). A deferential count of the leukocytes in the lung tissue from the male mice revealed a statistically significant difference between the female soiled bedding treated (n=5) and non-treated (n=5) groups (Fig. 3a). The prevalence of macrophages and neutrophils was higher in the BAL of male mice exposed to the female bedding; whereas, male mice kept in isolation from the female bedding showed a higher percent of lymphocytes in their BAL (Fig. 3b). Since neutrophils and macrophages are major effectors of the nonspecific immune defense against respiratory infections¹¹⁻¹³ and its migration to upper airway increase resistance of mice to experimental pneumonia¹⁴. That may help explain the observed enhancement of resistance to influenza in male mice exposed to the female soiled bedding.

Aerobic performance. Leukocyte migration into lung tissue is a common manifestation of respiratory airway inflammation, which entails a decline in the efficiency of both lung ventilation and aerobic performance^{15, 16}. But in our study, the average size of the leukocyte aggregations was smaller (11 – 1029 cells per aggregation) than has been reported for typical inflammatory infiltrations^{17, 18}. Also we determined the aerobic performance in the new set of male mice exposed (n=5) and not exposed (n=5) to female bedding. The cold-induced maximum oxygen consumption (MOC) was used as criteria of the aerobic performance. There were no statistically significant differences between the male mice exposed to female soiled bedding and

those not exposed (Fig. 4). Thus, the female scent induced increases in the number of small sized aggregations of leukocytes does not reduce the aerobic performance in male mice.

Thus, besides the well known induction of the neuroendocrine regulation of spermatogenesis and breeding behavior by female signals^{2,3,19}, this work demonstrates that such signals have additional role in increasing the leukocyte migration into the upper airways of male mice. Moving of the immunocompetent cells into the upper airways compensates the increased risk of respiratory infections, which accompanies the olfactory searching of a breeding partner. The proximate explanation of this phenomenon could be related with neuroendocrine effects of the female pheromones and neuroendocrine and immune links. Also we can not refuse non-pheromonal explanations such as an effect of the pathogen inhalation from contaminated soiled bedding. How it was found recently some bacterial compound might be detected by formil peptide receptors (FPR-rs) in vomeronasal olfactory epithelium²⁰. Our data is the first to demonstrate the migration of leukocytes in response to the naturalistic breeding signals that provided an anticipatory adaptation of male mice to potential risk of the respiratory infections.

METHODS

Female bedding treatment

Bedding (wooden flakes) from cages with mature BALB/c females were used for female scent treatment of males. Using 5 cages with 5 females per cage let as collected every day bedding that was soiled during the previous 5 days. The examination of vaginal smears revealed at least 30 % female in estrus. The soiled beddings or fresh wooden flakers were placed on the lid of male cages in wire mesh containers (about 5 ml) and were changed daily just before lights off. Four experiments have been done on BALB/c males that were kept one week before study with or without female scent.

Influenza A challenge

Mouse-adapted influenza virus A/WSN/33 (H1N1) was grown in embryonated chicken eggs. Virus containing allantoic fluid was harvested, and stored in aliquots at -80°C. Stock (5×10^7 FFU/ml) was serially ten-fold diluted by cold PBS.

Leukocyte trafficking into upper airways

Lungs samples were fixed in 10% buffered formalin. Histologic sections (4 μ m) of lung were stained with hematoxylin and eosin (HE). The number of leukocyte aggregations, their size, and their optical density in grey light were estimated in each section. Using regression equation, described previously we calculated number leukocytes per section²¹. Total leukocyte count in BAL was determined with a hemacytometer. For differential count of the leukocytes in BAL each samples (5 ml) were cytocentrifuged 5 min at 3g and 4°C. Then slices were stained by Wright-Giemsa.

Lung capacity

The maximum oxygen consumption (MOC) was assayed in male mice, which were treated either by female bedding or by fresh wooden flakes during week. Mice were exposed to HELOX (20 % O₂: 80 % He, +10°C) to stimulate the energy metabolism²².

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Author Contributions

E.A.L. measured the maximum cold-induced oxygen consumption; E.P.G. passaged the virus and infected mice; A.M.Z. made histological preparations of lung tissue; M.M.P. analyzed cells in BAL fluid. M.A.Z., M.M.P. and E.A.L. designed the study. E.A.L. and M.M.P. analyzed data and wrote the paper. All authors discussed the results and commented on the manuscript.

Author Information

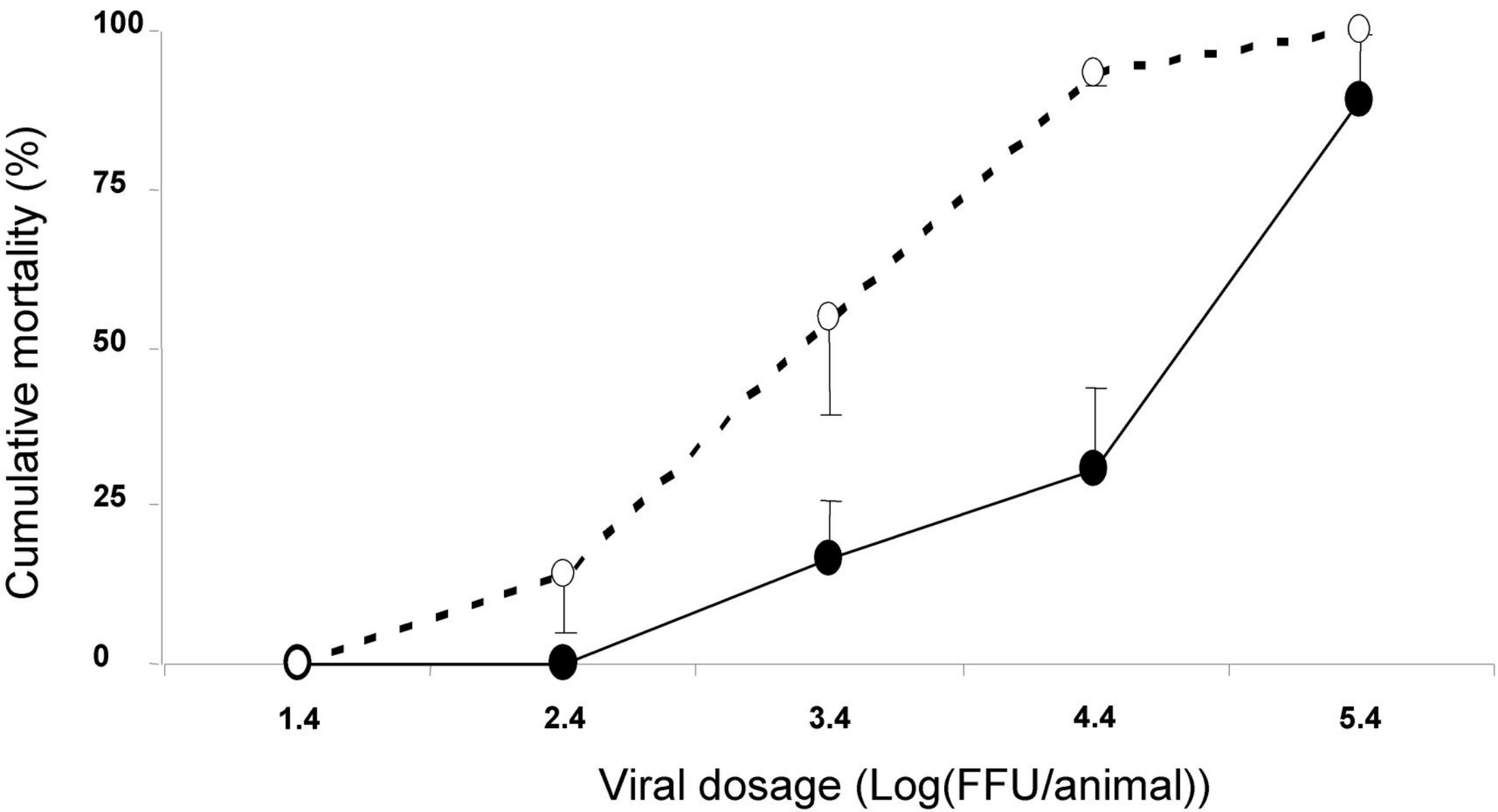
Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to E.A.L. (dimkit@ngs.ru) or M.M.P. (mmp@bionet.nsc.ru).

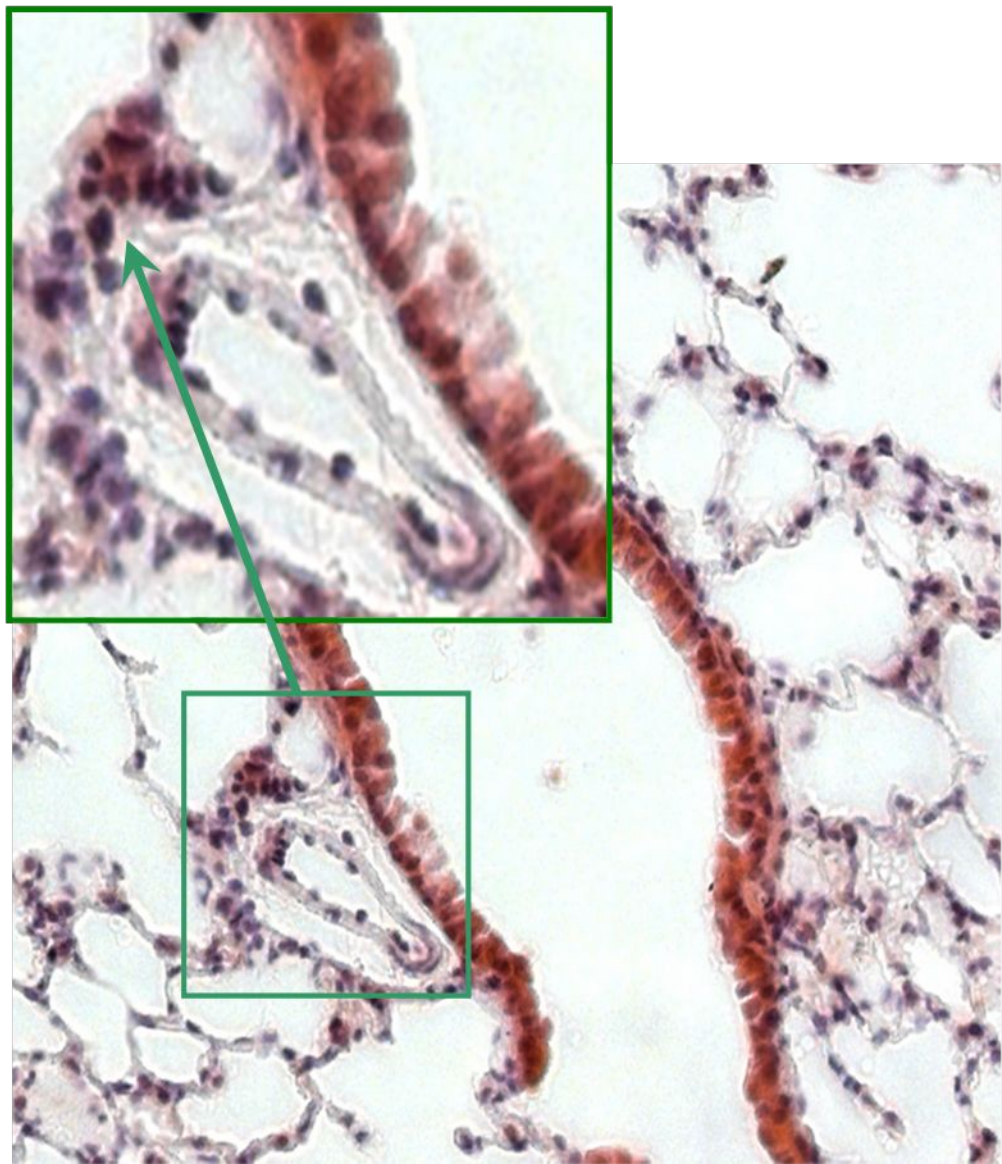
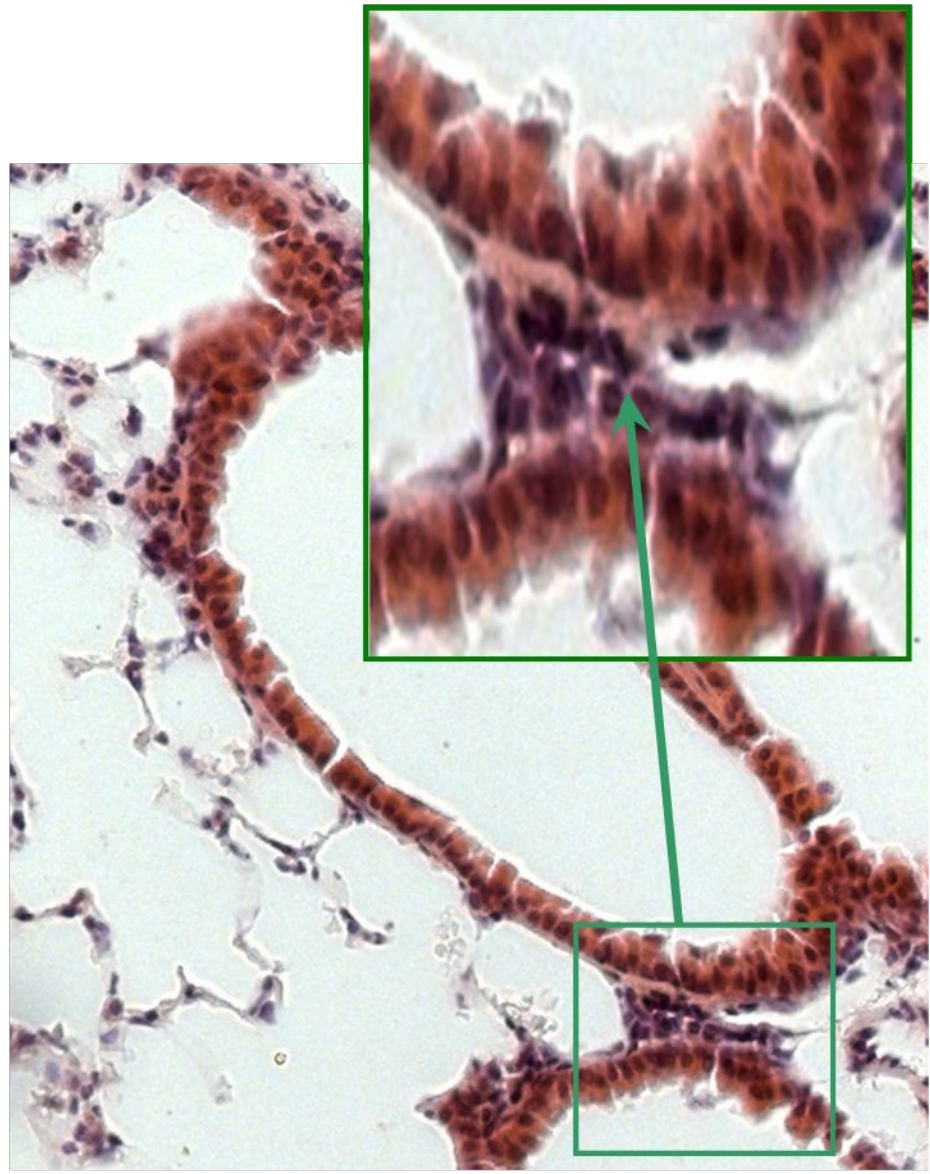
Figure 1 Cumulative mortality after challenge with influenza virus A/WSN/33 (H1N1). Non-exposed (white dots) and exposed to the female bedding (black dots) BALB/c male mice. LD₅₀ in males exposed to the female bedding (4.73±0.30 LogFFU) was significantly higher compare to males isolated from female bedding (3.28±0.35 LogFFU, $t=2.84$, $p<0.01$). Mean mortality±s.e.m. are shown.

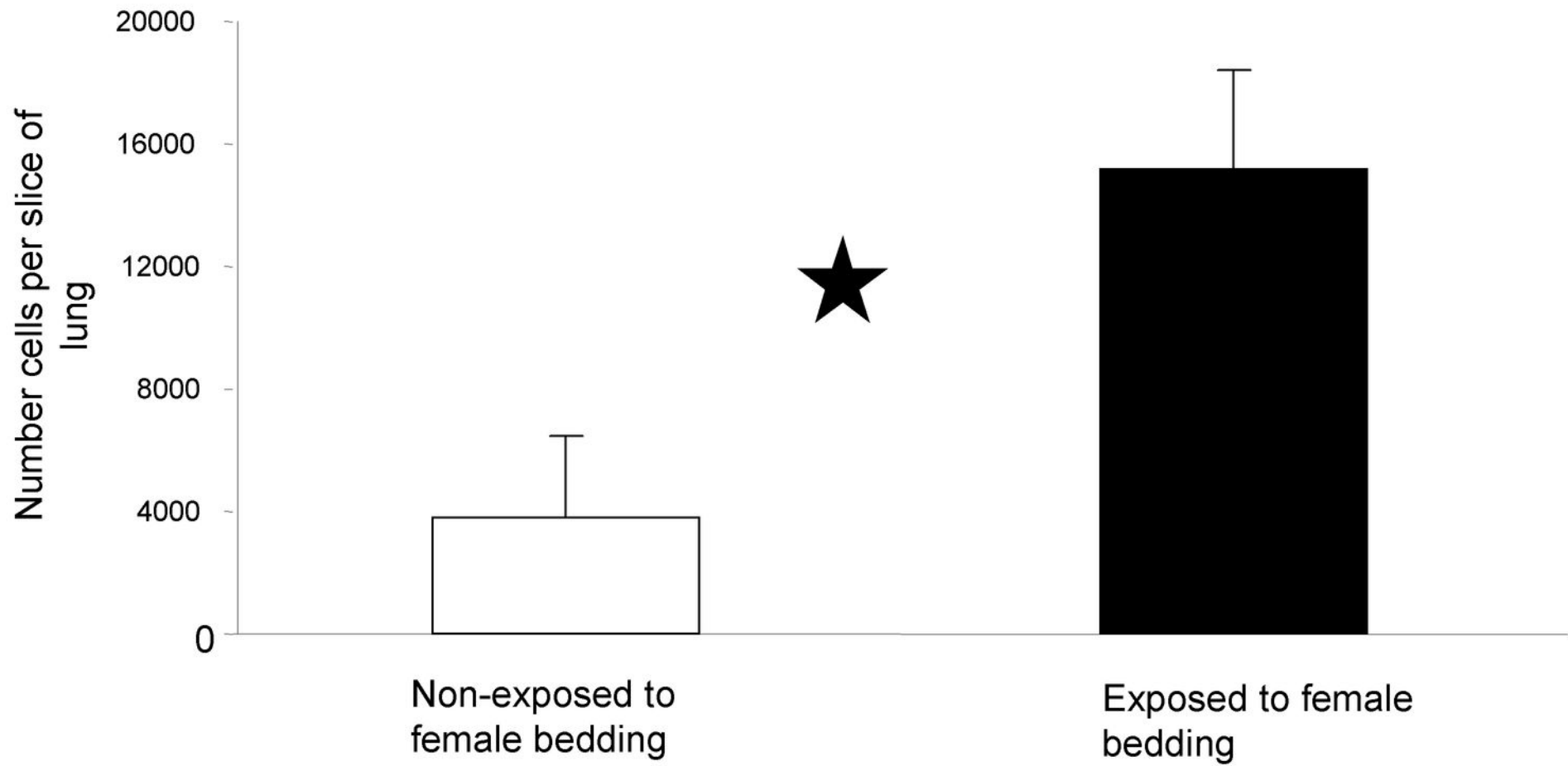
Figure 2 Leukocyte aggregations in lung tissue. **a**, Non-exposed to the female bedding BALB/c male mice. **b**, Exposed to the female bedding BALB/c male mice. Light microscopy (×10 and ×20 magnifications).

Figure 3 Number of leukocytes. **a**, Average number of leukocytes per histological slide in BALB/c male mice non-exposed (white) and exposed to the female bedding (black). Mean leukocytes±s.e.m. are shown; asterisks indicated $P=0.02$, $n=9$, $Z=2.32$. **b**, Percent of different types of leucocytes in broncho-alveolar lavage in BALB/c male mice non-exposed (right) and exposed to the female bedding (left). * indicated $P<0.01$, $n=10$, $Z=2.56$, † indicated $P<0.02$, $n=10$, $Z=2.45$, ‡ indicated $P<0.02$, $n=10$, $Z=2.45$.

Figure 4 Aerobic performance. Oxygen consumption during a 13 min exposure in HELOX by male mice that had been non-exposed (white dots) and exposed to the female bedding (black dots) (repeated measures ANOVA $F_{2,13}=1.92$, $p=0.39$). MOC was similar in males exposed ($n=8$, 9.73±0.39) and not exposed ($n=8$, 9.00±0.29) to the female bedding. Mean oxygen consumption±s.e.m. are shown.



a**b**

a**b**